DISSERTATION

CHEMICAL PRECEDENT STUDIES PROBING QUANTUM-MECHANICAL TUNNELING IN HYDROGEN ABSTRACTIONS FACILITATED BY COENZYME B_{12}

Submitted by

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In partial fulfillment of the requirements

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY KENNETH M. DOLL ENTITLED CHEMICAL PRECEDENT STUDIES PROBING QUANTUM-MECHANICAL TUNNELING IN HYDROGEN ABSTRACTIONS FACILITATED BY COENZYME B_{12} BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

CHEMICAL PRECEDENT STUDIES PROBING QUANTUM-MECHANICAL TUNNELING IN HYDROGEN ABSTRACTIONS FACILITATED BY COENZYME B_{12}

Recently, there have been numerous reports regarding the possible involvement of quantum mechanical tunneling in enzyme reactions that involve hydrogen transfer. The first chapter of this dissertation reviews the recent literature in this area, focusing on coenzyme B_{12} systems.

The next three chapters of this dissertation focus on the experimental quantification of an enzyme's involvement in tunneling, which can only be studied by a comparison of the same reaction both inside and outside of the enzyme. The solution thermolysis reaction of coenzyme B_{12} has been shown to cleanly generate adenosyl radicals that can abstract hydrogen from ethylene glycol, the same reaction that is observed in coenzyme B_{12}-dependent diol dehydratase. We have compared our solution data to the literature enzyme data and to another well studied enzyme, methylmalonyl-CoA mutase. In order to further our knowledge in this system, we have also synthesized

8-methoxyadenosylcobalamin, a methoxy analog of coenzyme B_{12}. This methoxy analog has demonstrated similar abstraction reactivity to coenzyme B_{12}, but with simpler reaction kinetics. A third system was also studied utilizing neopentyl cobalamin. In this system, we were able to monitor the thermolysis and abstraction reactions at lower, enzyme relevant temperatures. These systems have allowed us to conclude that in coenzyme B_{12} systems, enzymes may exploit quantum mechanical tunneling but have not evolved to enhance it.

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CHAPTER 1.

INTRODUCTION AND REVIEW OF RELEVANT LITERATURE

INTRODUCTION

Coenzyme B_{12} (Adenosylcobalamin, 5'-deoxy-adenosylcobalamin, AdoCbl) is a special enzymatic cofactor with the ability to perform in solution the same initial reactions that AdoCbl-dependent enzymes accomplish; the enzyme free rate is ~ 10^{12} times slower and there is no substrate specificity, however. This makes these systems valuable in chemical precedent studies where we can use AdoCbl and substrates to study the same reactions without the additional effects that enzymes induce. This dissertation will take advantage of enzyme-free AdoCbl reactions.

The format of each chapter of this dissertation follows the format required by the journal that it has been submitted to. More specifically, chapter two follows *J. Inorg. Biochem.* format, chapter three follows *J. Am. Chem. Soc.* format, and chapter four follows *Inorganic Chemistry* format. This chapter will also follow *Inorganic Chemistry* format.

This dissertation focuses on the homolytic cleavage of the Co–C bond and the subsequent hydrogen-abstraction reaction from substrate. This is a case with precedented quantum-mechanical (QM) tunneling behavior in enzyme reactions, ones that will be compared to our findings of QM tunneling in the same reactions outside of the enzyme.

This chapter will first give a brief historical overview of coenzyme B_{12}, then turn to a general overview of quantum-mechanical tunneling and the criteria used to diagnose such tunneling. QM tunneling in enzyme systems will be discussed next, including evidence for QM tunneling in AdoCbl-dependent systems.

Chapter two will be a presentation of the synthesis of an AdoCbl analog, 8-methoxy-5'-deoxy-adenosylcobalamin (8-MeOAdoCbl). This molecule proved to be

very valuable in our later studies allowing a more direct measure of the deuterium kinetic isotope effect (KIE) that is a diagnostic for QM tunneling.

Chapter three is the presentation of our studies using both the enzyme active AdoCbl and our synthetic 8-MeOAdoCbl analog. This, along with comparison to literature systems, allows us to draw conclusions about the ability of enzymes to exploit, but not enhance, QM tunneling.

Chapter four is the presentation of similar studies on another AdoCbl analog, neopentylcobalamin (NpCbl). NpCbl displays a Co–C bond cleavage reaction analogous to that seen in AdoCbl; hydrogen atom abstraction from substrate then also follows in this system. One difference in the NpCbl system, as compared to the earlier systems, is that these reactions can be studied at lower, enzyme-relevant temperatures. This allows us to directly compare the solution and the enzyme reactions without extrapolation. Additionally, this provides a third comparison along with the AdoCbl and 8-MeOAdoCbl systems. Chapter five is a summary of the dissertation in which further discussion and conclusions are presented.

GENERAL B_{12} LITERATURE OVERVIEW

Coenzyme B_{12} and methyl B_{12} (Figure 1.1; top) are essential cofactors in human life;[1] their cyano form is known as Vitamin B_{12}.

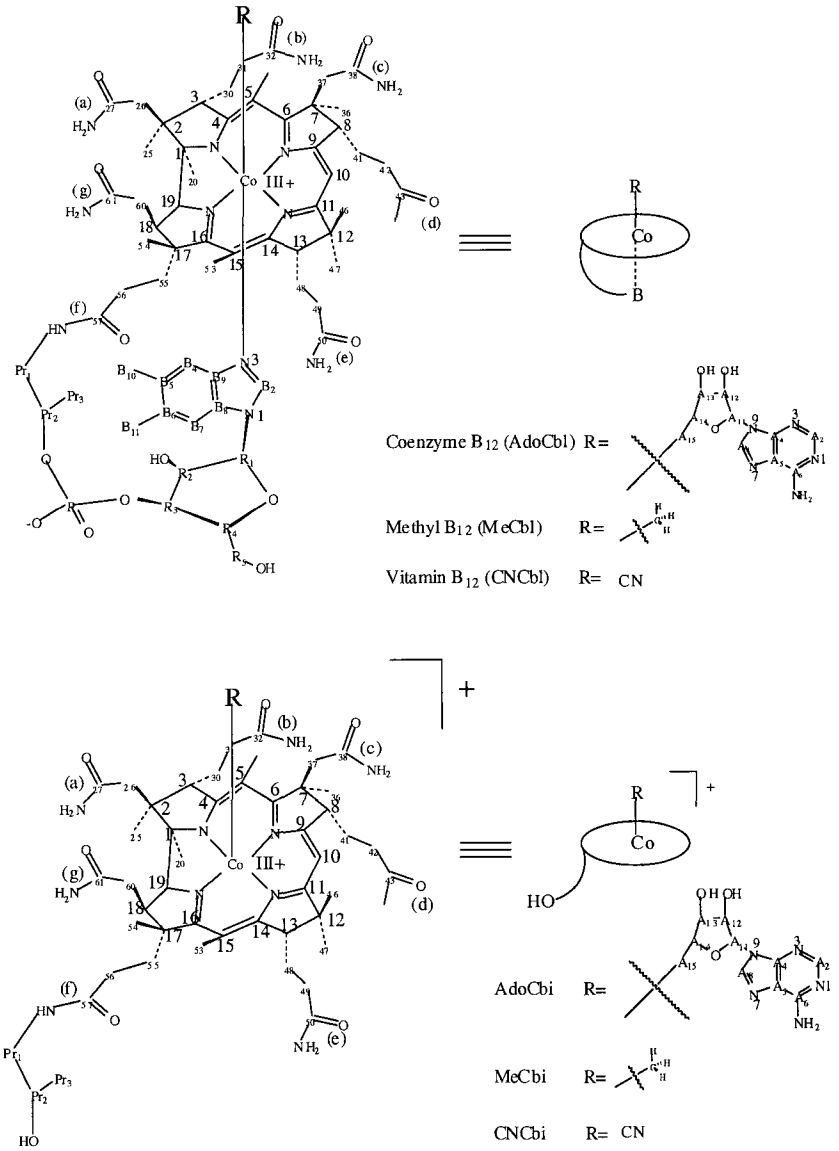


Figure 1.1

The structure of cobalamins (top) and cobinamides (bottom) using IUPAC nomenclature.

Coenzyme B_{12} (Adenosylcobalamin, AdoCbl) is required in the metabolism of fatty acids into the tricarboxylic acid cycle by the enzyme l-methylmalonyl-CoA Mutase (Figure 1.2)[1,2]. Deficiencies in AdoCbl lead to increased levels of D-methyl-malonic acid [1,3,4] and 2-methylcitric acid[1,5].

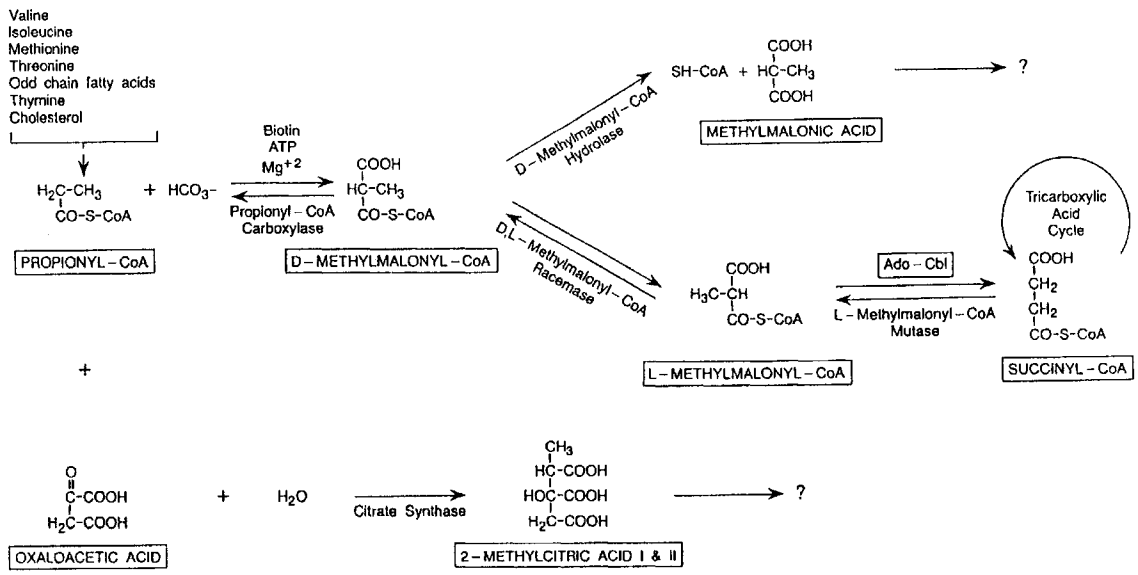


Figure 1.2

A scheme of an essential reaction pathway which is regulated in by AdoCbl in mammals. (Adapted from Stabler in Banerjee, R.; Editor *Chemistry and Biochemistry of B_{12}* 1999).

Methyl B_{12} (Methylcobalamin, MeCbl) is an essential cofactor in the conversion of homocysteine to methionine (Figure 1.3). Deficiencies in MeCbl lead to elevated levels of homocysteine as well as possible lowered levels of S-adenosyl methionine, species which both have been implicated as potential factors in heart disease.

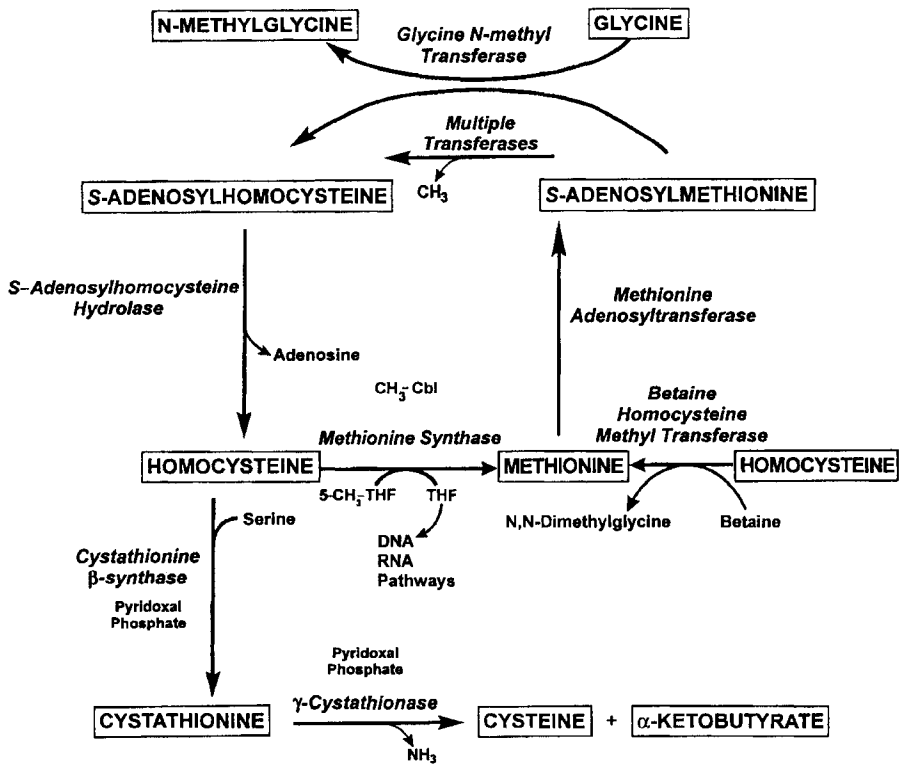


Figure 1.3

A scheme of an essential reaction pathway which is regulated in by MeCbl in mammals. (Adapted from Stabler in Banerjee, R.; Editor *Chemistry and Biochemistry of B_{12}* 1999).

Lack of the "intrinsic factor" that transports cobalamins is the cause of pernicious anemia, a potentially fatal disorder. The first effective treatment of pernicious anemia was the 1934 Nobel prize winning liver diet prescribed by George Whipple, George Minot and William Murphy.[6,7] Cobalamin deficiencies cause megaloblastic anemia[1,8] that can lead to blood and bone marrow abnormalities. Cobalamin deficiencies can also lead to a spongy degeneration of the myelin sheaths in the spinal cord[8] leading to symptoms such as parethesais[9] and pain sensations. Cobalamin deficiency has even been mentioned as a possible factor in the cause of schizophrenia.[10] Due to its biological and chemical significance, the study of cobalamins and cobalamin dependent enzymes continues to be of significant interest.

There are 17 different AdoCbl-dependent enzymes that have been discovered, 11 of which catalyze a 1,2-hydrogen / R shift; the remaining 6 reactions reduce ribonucleotides to deoxyribonucleotides. The 11 1,2-hydrogen / R shift enzymes share a common general scheme (Figure 1.4): First the Co–C bond cleavage produces an adenosyl radical (Ado$^{\bullet}$), then this radical abstracts a hydrogen atom from the substrate which leads to further chemistry (a more detailed discussion of the mechanism for individual enzymes is presented later in this chapter).

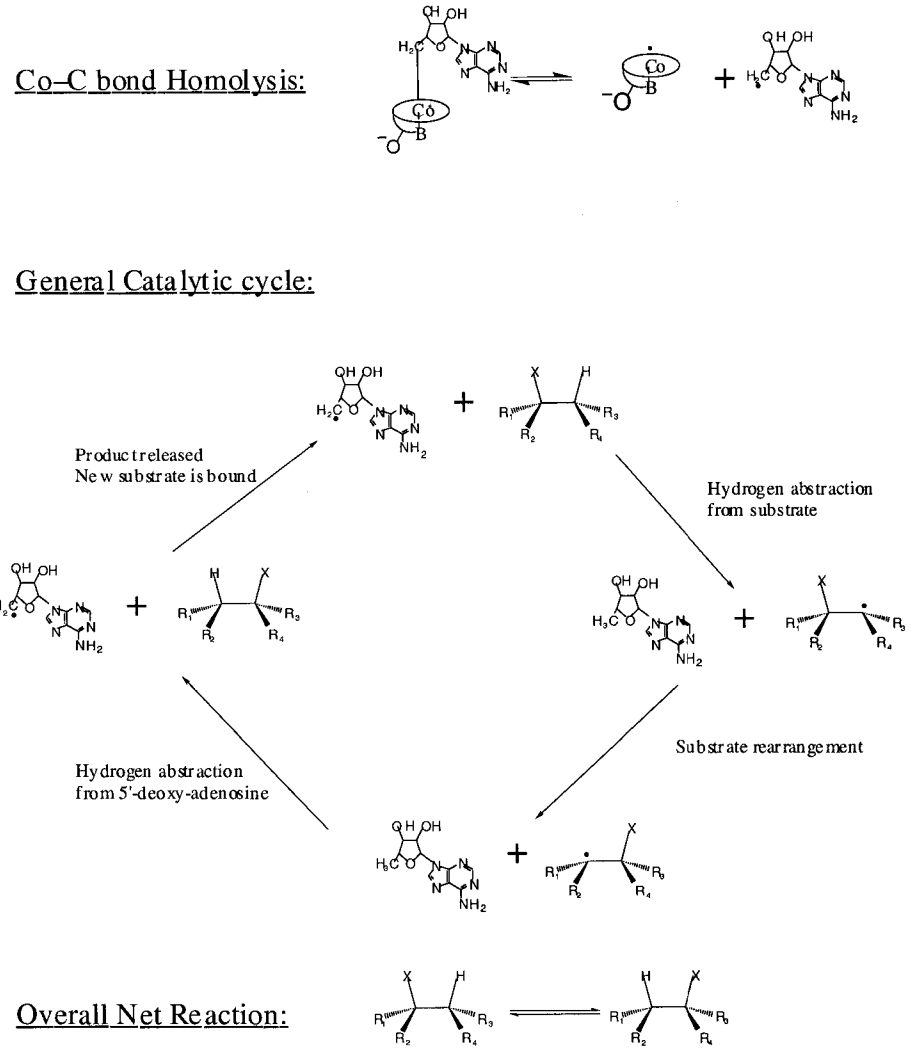


Figure 1.4

The proposed general mechanistic cycle for many AdoCbl dependent enzymes. The enzyme catalyzes the cleavage of the Co–C bond which produces a relatively stable Co(II)Cbl$^{\bullet}$ and an Ado$^{\bullet}$ which is capable of hydrogen atom abstraction from many substrates. The enzyme protects the reactive radical intermediates and may assist in the substrate rearrangement in special cases.

Vitamin B_{12} was first isolated in 1948 by Folkers and co-workers.[11,12] Its name, a previously unused continuation of the B series,[12] was intended to portray nutritional significance and was also recommend by Smith and Parker.[13] Its X-ray crystal structure was determined by Hodgkin[14] in 1955, earning her the Nobel prize in chemistry in 1964. An active form of the vitamin, Coenzyme B_{12}, was first discovered in 1958 and identified as similar to Vitamin B_{12} by Barker and Weissbach.[15] In 1961, its X-ray structure was also solved by Hodgkin.[16] In 1972, Woodward and Eschenmoser[17-20] completed the 37 step synthesis of the Vitamin B_{12} precursor, cobyric acid from which, the four step synthesis of Vitamin B_{12} had been previously accomplished by Freidrich[21] This work was a key in the development of the Woodward-Hoffman rules on cycloaddition leading to Woodward's Nobel prize in chemistry in 1965. The biosynthesis of cobalamins has also been studied[20,22] with the discovery of both aerobic[23,24] and anaerobic[25] pathways.[1,26] Both systems involve an interesting ring contraction from a reduced porphyrin to a corrin in two or more reaction steps. Overall, cobalamins have a rich history, being the largest crystal structure in 1955,[14] the first naturally discovered organometallic molecule,[27] and the subject of early tests of powerful and now common physical tools such as HPLC and FABMS.

The overall structure of cobalamins is shown in Figure 1.1 (top), using nomenclature that was standardized by IUPAC-IUB in 1964 and clarified in 1973. The name cobalamin is derived from cobalt, and the name corrin is derived from "core."[28] The structure of a cobalamin consists of a corrin ring, a 19-carbon cyclic ring surrounding 4 nitrogen atoms. This structure is similar to a porphyrin, with two important exceptions: first is a missing methine carbon between carbons 1 and 19, which

causes an upward folding of the ring. Second, four of the double bonds in a porphyrin are saturated in a corrin, leading to a formal charge of 1- for a corrin. Located between the nitrogen atoms is a cobalt atom, which is usually in the low spin, 3+ oxidation state in solid cobalamins; the 2+ and 1+ oxidation states of cobalt are accessible in solution. There are 7 methyl groups attached to the ring, 5 of which are in the β position relative to a double bond. A total of 7 amide side chains on the corrin ring, labeled a-g, are also present. Chains a, c, and g are orientated toward the top or β side of the corrin ring and the other 4 side chains are orientated toward the bottom, or α side of the corrin. Chain f has a β-5,6-dimethylbenzimidazol moiety attached to the amide through a phospodiester linkage. Nitrogen 3 of the dimethylbenzimidazole moiety coordinates to the cobalt atom in the solid phase, and in solution at neutral or basic pH.

One important class of cobalamin analogs called cobinamides (Cbi) are missing this axial ligand and phosphate linker (Figure 1.1 (bottom)). Cobalamins almost always have a ligand coordinated to the cobalt on the β side of the corrin ring. In AdoCbl, this key ligand is 5'-deoxyadenosine, one shown by molecular mechanics and 2 dimensional NMR experiments[29] to have the lowest energy conformation with the adenosyl ligand over the "south" portion of the corrin ring (over carbons 12-15).[30]

The most important structural characteristic of AdoCbl is its Co–C bond, a novel biological bond, one postulated to be important as early as 1961[16,27,28] and later shown to be the source of the reactivity of AdoCbl in diol dehydratase.[31-36] In diol dehydratase, the AdoCbl acts as a source of Adenosyl radicals (Ado•) which do subsequent chemistry; Co–C bond homolysis is involved in the mechanisms of other enzymes as well, including methylmalonyl-CoA mutase,[37-39] glutamate mutase,[40-42] ethanolamine ammonia lyase,[43-45]

and ribonucleoside triphosphate reductase. The proposed mechanisms for these enzymatic systems will be discussed in more detail later in this chapter.[46-48]

Because the Co–C bond is contained in the cofactor and not in the enzyme, *the study of AdoCbl in a simple solution thermolysis reactions provides a valuable comparison point for the enzyme reaction.* The activation parameters of the Co–C bond homolysis were measured in aqueous solution,[49] as well as in ethylene glycol solution (Figure 1.5)[50,51] by Finke and Hay. The Co–C bond was surprisingly stable, with a Co–C bond dissociation energy of 30 ± 2 kcal mol^{-1}. (The same bond dissociation energy was measured by Halpern to be 26 ± 2 kcal mol^{-1} in a system where a Co–C heterolysis side reaction was later shown to be present.)[52]

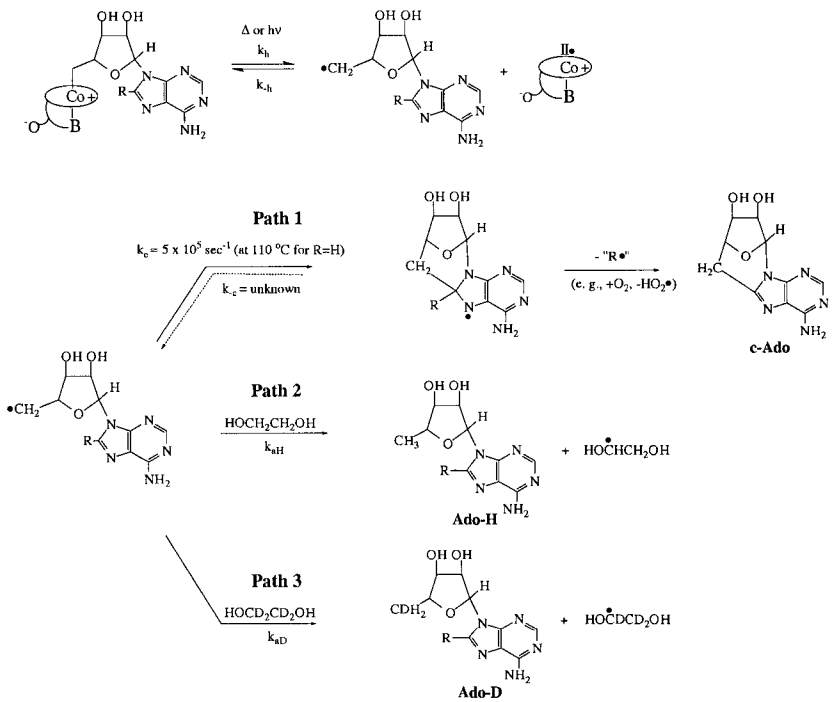


Figure 1.5

The reaction of AdoCbl in ethylene glycol solution.

The strength of the Co–C bond has also been studied by density functional theory,[53-58] giving a value of 33 ± 2 kcal mol^{-1} (in the gas phase). When these systems are compared with diol-dehydratase Co–C bond cleavage data,[34-36] a rate enhancement factor of ~ 10^{12} can be calculated for the homolysis of the Co–C bond. This enzymic rate enhancement was a major focus of AdoCbl research for many years, and is still not well understood.

Early work in the study of the Co–C bond activation rate enhancement focused on the dimethylbenzimidazole axial base. This base has been shown by crystal structures to be bound to the Co atom in some AdoCbl dependent enzymes (Class I) but unbound in others with, instead, the imidazole side chain of a histidine coordinated to the cobalt atom (Class II) (vide infra). An accurate measurement of the Co–C bond strength in adenosylcobinamide ($AdoCbi^+$) (an analog of AdoCbl that does not contain the axial base) was first accomplished by Finke and Hay in 1987.[59] The results show a Co–C bond dissociation energy of 34.5 ± 1.8 kcal mol^{-1}. Although this is 4.5 ± 2.7 kcal mol^{-1} stronger than the Co–C bond of a cobalamin, it shows that the axial base coordination is only enough to account for a ~ 10^2 fold enhancement of the Co–C bond cleavage rate, only a small part of the ~10^{12} fold acceleration seen in the AdoCbl-dependent enzymes. The possibility that the enzymes could be using the histidine residue to weaken the Co–C bond has been studied by chemical precedent[60-64] and theoretical methods. One interesting hypothesis was the so-called steric "mechanochemical trigger mechanism" (also called a "butterfly effect"),[65,66] in which the enzyme forces the histidine residue close to the corrin, increasing the corrin folding, thereby weakening the Co–C bond. The study of the butterfly effect has been the subject of molecular modeling and molecular dynamics[29,30,65,67-69] as well as density functional theory studies.[55,70] Overall, the steric

effect has been shown to contribute only an estimated 3 kcal mol^{-1} of destabilization to the Co–C bond.[68,71]

It is, however, possible that the axial base is controlling the mode of cleavage in the Co–C bond (i.e., homolysis vs heterolysis), and there is evidence for this from both experiments and molecular orbital theory.[72] Hence, the binding constants of cobinamides [AdoCbi$^+$, methyl cobinamide (MeCbi$^+$), neopentyl cobinamide (NpCbi$^+$)] with exogenous bases (such as pyridines, imidazoles, anilines, cyanide, and azide) have been studied.[60-64,73-75] The kinetic products produced by the thermolysis of these reactions lead to the conclusion that a short Co–N bond distance or a strong σ-electron donor such as CN^- causes a large increase in the amount of abiological heterolysis,[61-64] while a longer Co–N bond distance show more of the biologically relevant homolysis. In work that is still controversial, Sirovatka and Finke have shown that both the σ-electron donation and the π-electron donation are important in the determination of the final reaction pathway, with the study of a series of sterically hindered and unhindered imidazoles [for further discussion on this topic, see Appendix D (Doll, K. M.; Finke, R. G. to be submitted)].[63,64,76]

Electron transfer is another hypothesis which has been checked as a possible explanation for the enzymic acceleration of 10^{12}, that is, reduction of the Co–C bond by 1 electron, which would populate an anti-bonding orbital and reduce the bond order of the Co–C bond order to 1/2. The bond strength of the $1e^-$ reduced MeCbl$\bullet^-$ system was measured by Martin and Finke.[77,78] It was found that the bond strength is lowered from 37 ± 3 kcal mol^{-1} to only ~12 kcal mol^{-1}. This *could*, therefore, account for a 10^{15} fold acceleration in homolysis rate. However, even this large rate acceleration is insufficient

to overcome the required pre-equilibrium electron transfer. Accounting for a thermodynamically uphill electron transfer pre-equilibrium predicts a necessary 10^{23} fold rate enhancement of the homolysis to get the observed 10^{12} fold effect. This would force the rate constant $k_h > 10^{11}\ s^{-1}$, approaching the vibrational timescale and, therefore, is physically improbable.[78-81] Another strong piece of evidence against the electron transfer hypothesis is that the reduction potential of MeCbl is highly negative in aqueous solution (~–1.6 V vs SCE at –30 °C).[82] There is no reductant with that potential available in any known AdoCbl-dependent or other biological system. The lack of any observable Co(I)Cbl (and Me•) was also cited as evidence against electron transfer.

NMR has been another powerful method for the study of cobalamins. Early NMR studies of AdoCbl was performed by Pratt[83,84] starting in 1965. The complete ^1H and ^{13}C NMR assignments of AdoCbl were reported in 1986 using 2-dimentional NMR techniques.[85] Those NMR studies were followed by the NMR solution structure of $AdoCbi^+$ in 1989.[86] Overall, the complete ^1H and ^{13}C NMR structures of at least 32 AdoCbl analogs have been determined, mostly by the Marzilli[72,86] and Brown[1,29,87] labs. One seemingly obvious insight from cobalamin NMR is that although a cobalamins contains ≥ 88 protons, the aromatic region of the ^1H NMR is not crowded, allowing for identification and purity assessment of cobalamins in a simple NMR experiment.[88]

Dozens of crystal structures[1,26,89-94] of cobalamins and cobalamin analogs have followed the original crystallographic work of Hodgkin.[14,16,95-100] Kratky has attempted to correlate the length of the axial base Co–N bond with the Co–C bond length (Figure 1.6) and the corrin fold angle by using data from 17 different crystal structures.[1,26] The fold angle shows a significant amount of correlation, with increased folding as the Co–N

distance becomes shorter. In addition, reducing the size of the axial base as in cyano-imidazolylcobamide yields a small upward folding angle.[90] It is also of note that Co(II)cobalamin, with no β-axial ligand follows this same correlation with a fold angle of 16.3° and a Co–N bond length of 2.13 Å.[89]

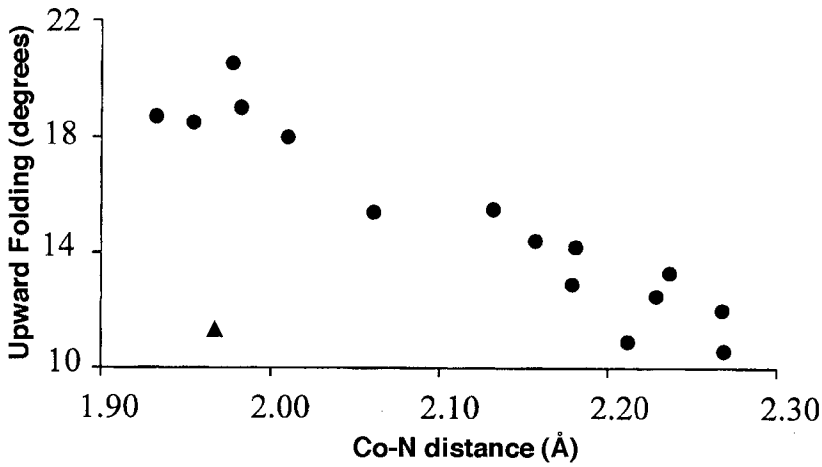


Figure 1.6

A correlation between upward folding angle and Co–N bond length from crystal structure data. The data point marked▲ is for the sterically smaller axial base, imidazoylcobamide. (Adapted from Kratky in Krautler, B.; Arigoni, D.; Golding, B. T.; Editors *Vitamin B_{12} and B_{12}-Proteins.* 1998).

The Co–C bond length has also been correlated with Co–N bond length by Burgi (Figure 1.7),[1,101] with the discovery of a so called "anomalous trans influence" where shortening the cobalt bond to one axial ligand *shortens* the bond to the other axial ligand. This trans influence has also been studied by density functional theory.[102]

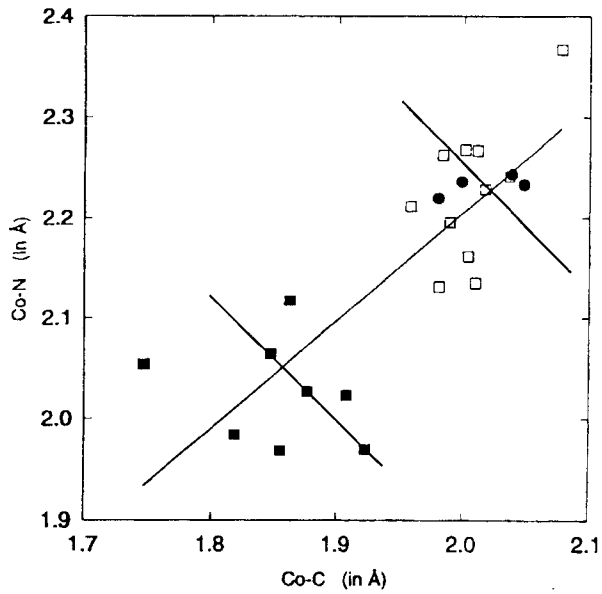


Figure 1.7

A "correlation" between Co–C bond length and Co–N bond length from crystal structure data. The symbol differentiate the top β ligand (■: β ligand is CN, ●: β ligand is Ado, □ other β ligand). Note, however, that these data can also be "correlated" by two separate lines nearly perpendicular to the Bürgi's correlation. (Adapted from De Rider, D. J. A.; Zangrando, E.; Bürgi, H-B. *J. Mol. Struct.* **1996**, *374*, 63).

It is also of interest that in a thiolatocobalamin where the axial ligand is a sulfur-bound thiol,[91,92,103,104] the Co–S bond seems to have a normal trans effect on the Co–N bond based on 3 X-ray crystal structures and density functional theory (Figure 1.8).[58]

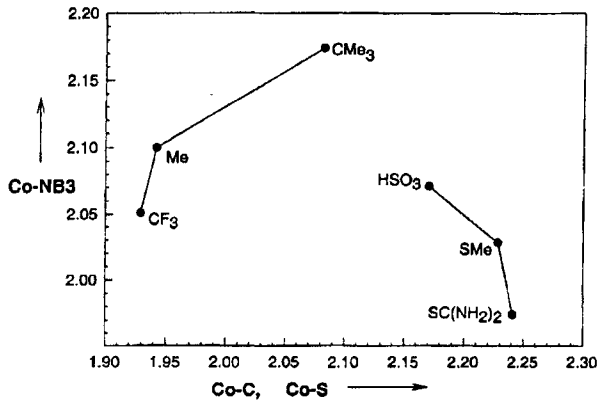


Figure 1.8

A correlation between Co–C or Co–S bond length and Co–N bond length from density functional calculations; the data also show close agreement with crystal structure data (not shown). (Adapted from Randaccio, L.; Geremia, S.; Stener, M.; Toffoli, D.; Zangrando, E. *European Journal of Inorganic Chemistry* **2002**, 93).

Unfortunately, at this time, there is not a single crystal structures of any cobinamide. One possible explanation is that the missing phosphate linker causes a cobinamide to have an overall 1+ charge. This charge must be compensated for by a counter anion, perhaps causing difficulty in crystallization. Cobalamin analogs where the axial base is cleaved, but the phosphate remains, have been studied as a possible remedy to this problem by both Finke and White,[105] and Toraya[106] but crystal structures or even evidence of diffracting crystals has not been published.

Significant insight into the 10^{12} fold enzymatic acceleration of the Co–C bond cleavage has come from the recent crystal structures of the AdoCbl dependent enzymes. There are now crystal structures for 4 different AdoCbl dependent enzymes and the cobalamin binding domain of 1 MeCbl dependent enzyme (Table 1.1). These crystal structures have helped to characterize the binding of AdoCbl. In some cases, comparisons of the apoenzyme and holoenzyme have been made giving insight into the Co–C bond activation.

Table 1.1

Reported crystal structures of AdoCbl and MeCbl dependent enzymes.

Enzyme	Base on/off	Size (kDa)	Notes
Methionine Synthase (MetH)[107] (MeCbl dependent)	Off	136 monomer with 4 domains	The 27 kDa binding domain bound to MeCbl was crystallized. Discovery of the ligand triad.
Methylmalonyl CoA Mutase (MMCoA)[108,109]	Off	150 dimer $\alpha\beta$	Crystallized with AdoCbl both substrate free and with a substrate mimic desulpho-CoA. A large conformational change, which may cause enzymatic cleavage of the Co–C bond, is observed.
Glutamate Mutase (GM)[110]	Off	136 tetramer $\varepsilon_2\sigma_2$	ε subunits each contain a AdoCbl binding site. Crystallized with CNCbl and MeCbl. Crystallized with inhibitor (2S,3S)-tartrate

Diol Dehydratase (DD)[111,112]	On	220 dimeric trimer $(\alpha\beta\chi)_2$	Cobalamin bound between the α and β subunits. Crystallized with adeninylpentylcobalamin and CNCbl with 1,2 propane diol substrate. Discovery of a potassium assisted hydroxyl migration.
Ribonucleotide triphosphate reductase (RTPR)[113]	On	82 monomer	Allosterism in a monomeric enzyme. Contains a $(\alpha/\beta)_{10}$ barrel over the cobalamin in the active site, not the common TIM barrel. Crystallized with adeninylpentylcobalamin bound and in the apoenzyme form.

The earliest report of an AdoCbl-dependent enzyme crystal structure was of methylmalonyl-CoA mutase (MMCoA) from *Propionibacterium shermanii* in 1996.[108] MMCoA catalyses the rearrangement of succinyl-CoA into methylmalonyl-CoA (Figure 1.9).[1] As was previously suspected from earlier EPR experiments,[114] the crystal structure showed that the dimethylbenzimidazole is not coordinated to the cobalt but has been replaced with the imidazole side chain of a histidine residue with a Co–N bond length of ~ 2.5 Å.[108] This was the first conclusive evidence of a Class II base-off AdoCbl dependent enzyme (Recall that Class II is where the cofactor is bound with the axial base not coordinated to the cobalt.) The top of the corrin is protected by a $(\beta/\alpha)_8$ triosephosphate isomerase (TIM) barrel in its binding site, a motif seen in every cobalamin-dependent enzyme crystal structure until the recent ribonucleoside triphosphate reductase structure. (This structure instead has a $(\beta/\alpha)_{10}$ barrel which is required for allosteric regulation, vide infra.) Subsequent crystal structures of MMCoA mutase[109] bound with a substrate (succinyl-CoA) or an inhibitor (2-carboxypropyl-CoA and 3-carboxypropyl-CoA) clearly show a significant change in the conformation of the protein when compared with a structure of a protein with an open active site.[115] The substrate binding causes the active site to close and drives the adenosyl ligand away from the cobalt atom via a steric interaction. This distorts the coordination sphere around the cobalt atom and weakens the Co–C bond, leading the enzyme to be labeled a "molecular nutcracker".[1] Similar modes of action are suspected in other AdoCbl dependent enzymes such as diol dehydratase and glutamate mutase. Kinetic isotope effect studies and evidence for quantum mechanical tunneling in this system will be discussed later in this chapter and in a detailed discussion in chapter 3.

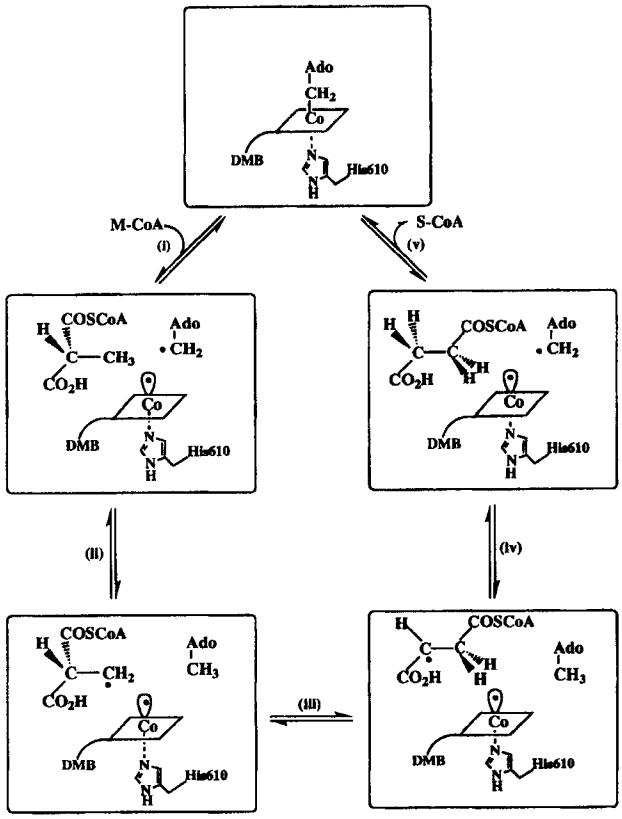


Figure 1.9

The proposed mechanistic scheme for the rearrangement of Methylmalonyl-CoA to Succinyl-CoA catalyzed by AdoCbl-dependent MMCoA mutase. (Adapted Padmakumar, R.; Padmakumar, R.; Banerjee, R. *Biochemistry* **1997**, *36*, 3713).

Diol-dehydratase (DD) is an enzyme that converts diols into aldehydes plus water. Toraya reported the crystal structure of diol dehydratase from *Klebsiella oxytoca* bound to the inactive cofactor cyanocobalamin[111] in 1999 and bound to the also inactive cofactor, adeninylpentylcobalamin,[112] in 2000. These crystal structures support Toraya's mechanism for diol dehydratase (Figure 1.10)[33,111,116-118] which involves potassium ion assistance in a hydroxyl radical migration. This mechanism is different than Ables' earlier proposed mechanism in which the cobalt atom participates in the stabilization of the substrate.[27] Early evidence against participation of the cobalt atom along the reaction pathway was provided by Finke and Schiraldi with the synthesis of possible Co-substrate intermediates, work which showed that non-enzymatic products were produced from the putative Co-bound intermediates.[119] Kinetic isotope effect studies, and evidence for quantum mechanical tunneling in diol dehydratase will be discussed later in this chapter and in more detail in chapter 3.

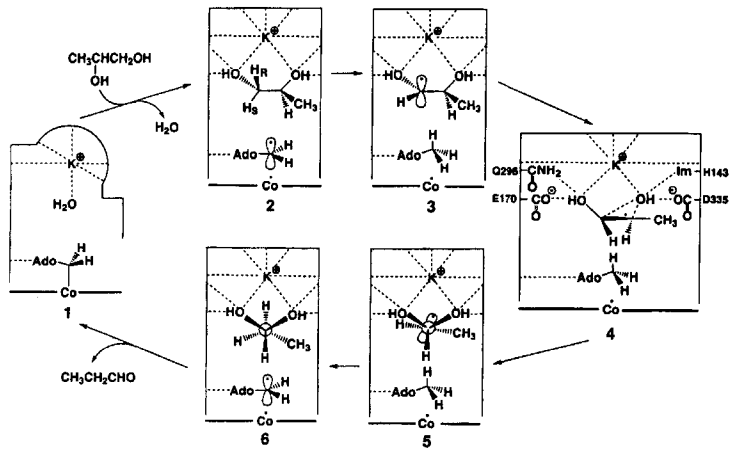


Figure 1.10

The proposed mechanistic scheme for the K^+ ion assisted hydroxyl migration in the diol dehydratase reaction with 1,2-propane diol as substrate. (Adapted Toraya, T. *J. Mol. Catal. B: Enzym.* **2000**, *10*, 87).

Glutamate Mutase is an AdoCbl dependent enzyme that catalyzes the reaction of L-glutamate into L-threo-3-methylasparatate with a proposed mechanism involving a hydrogen abstraction followed by a carbon skeleton rearrangement (Figure 1.11).[40,120] The crystal structures of glutamate mutase from *Clostridium cochlearium* bound to inactive cofactors, MeCbl and CNCbl, and with (2S,3S)-tartrate bound as substrate were reported in 1999.[110] They show 5,6-dimethyl-benzimidazole base-off coordination to cobalamin similar to MMCoA mutase, with a histidine imidazole coordinated at a distance of ~ 2.3 Å.[110] The crystal structure also provides further evidence for a conformation change with substrate binding, with at least 14 hydrogen bonds between the enzyme and the substrate being detected. The non-participation of the cobalt in the radical rearrangement is also supported by the large distance from the cobalt atom to the substrate. Kinetic isotope effect studies and evidence for quantum mechanical tunneling in the case of glutamate mutase will be discussed later in this chapter.

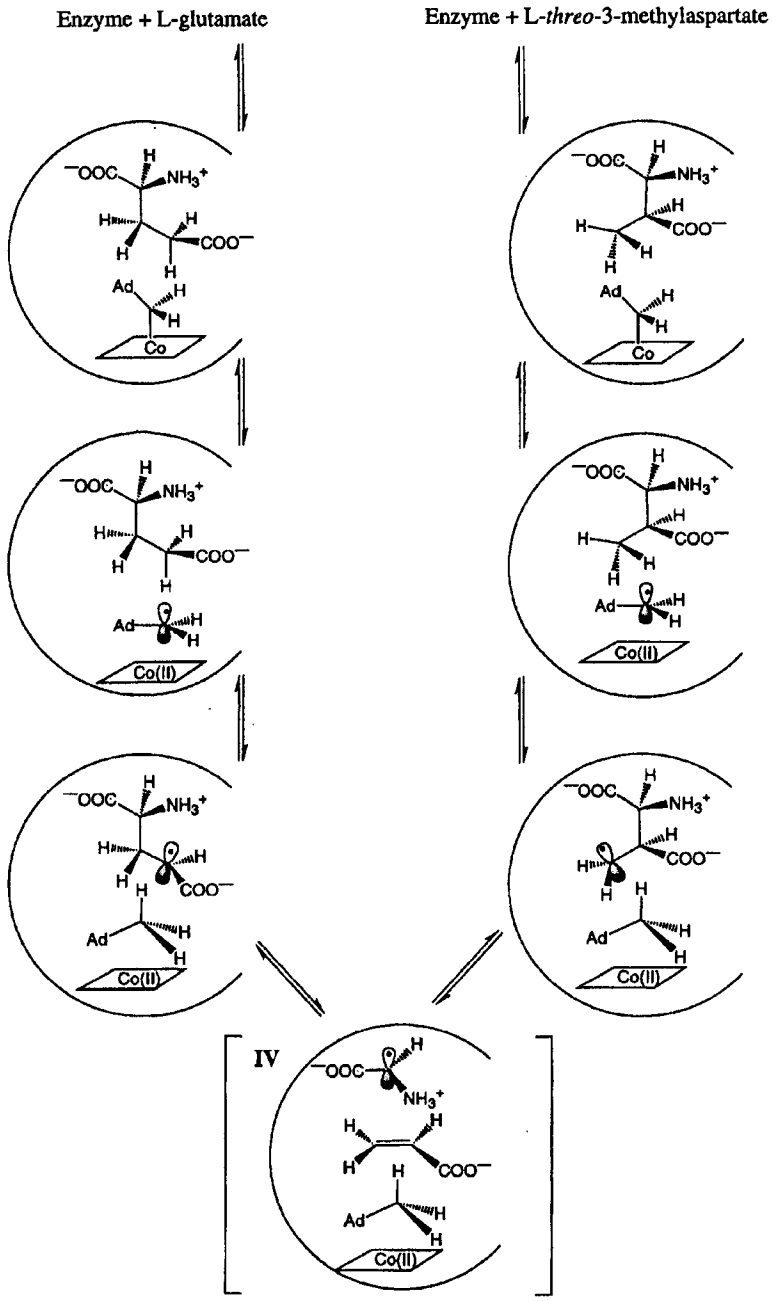


Figure 1.11

The proposed radical fragmentation/recombination mechanistic scheme for the rearrangement of L-glutamate to L-threo-3-methylaspartate catalyzed by Glutamate Mutase (Adapted Marsh, E. N. G.; Ballou, D. P. *Biochemistry* **1998**, *37*, 11864).

Ribonucleoside triphosphate reductase (RTPR) is an enzyme which converts ribonucleoside triphosphates to deoxyribonucleoside triphosphates. Class II ribonucleotide reductases (RNRs) use AdoCbl in order to generate an Ado$^{\bullet}$, which then abstracts a hydrogen atom from cysteine 408 to generate a thiyl radical. The thiyl radical is the species which does the hydrogen atom abstraction from the substrate (Figure 1.12). There is now considerable evidence that the Co–C cleaveage, the S–H cleavage, and the Ado–H formation reactions are effectively concerted or nearly so.[46-48,66,121,122] The deuterium kinetic isotope effect on this reaction has been measured using 5'-$^{2}H_2$-AdoCbl in D_2O, and is only 2.7 ± 0.3.[121] The question of why the enzyme uses a cysteine radical has been studied by the synthesis of thiolatocobalamins which contain a Co–S bond, by both the Finke[92,103,104] and Randaccio groups.[58,91] Although some factors that influence thiolatocobalamin stability have been studied, the measurement of the Co–S bond strength remains unreported and is still of considerable interest.

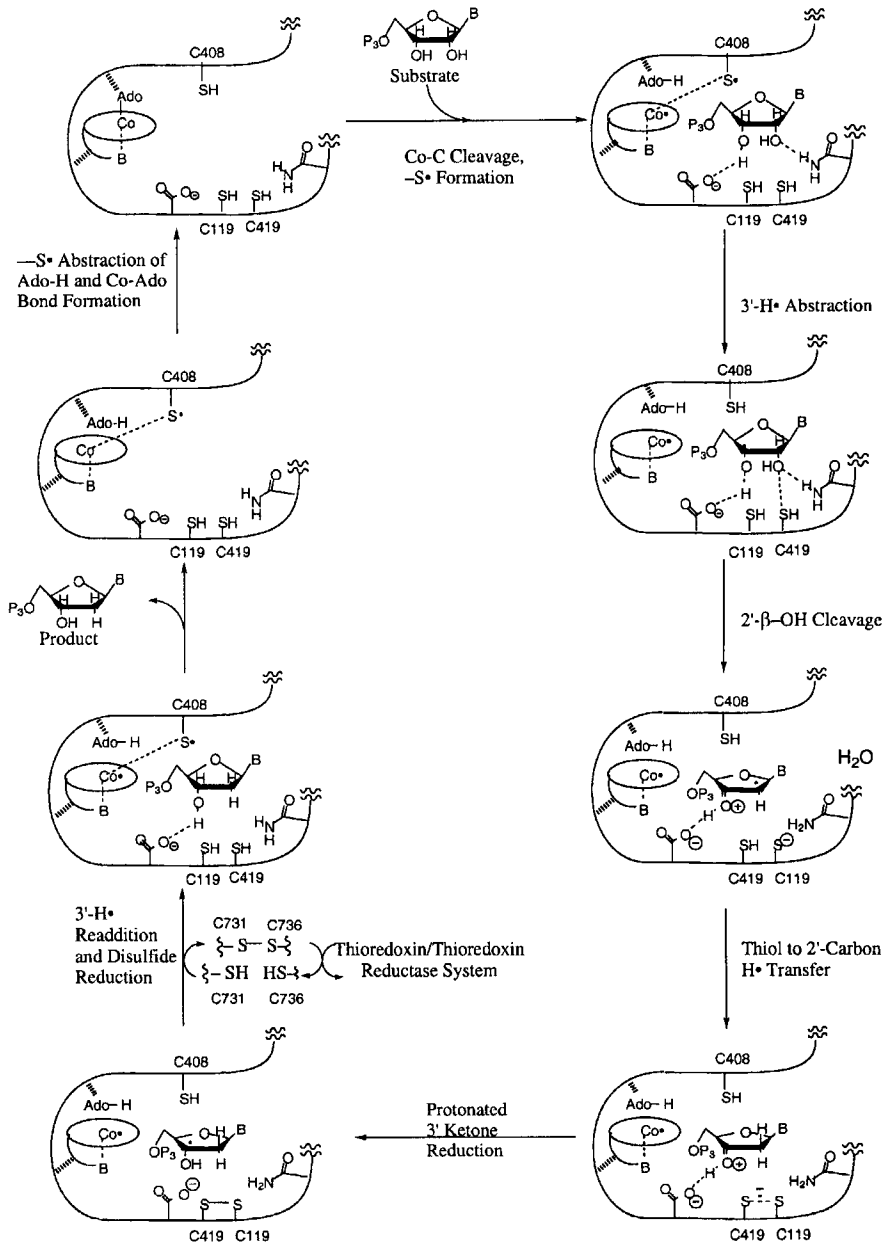


Figure 1.12

One proposed mechanism of RTPR from *Lactobacillus leichmanni*. (Adapted from Finke in Krautler, B.; Arigoni, D.; Golding, B. T.; Editors *Vitamin B_{12} and B_{12}-Proteins*. 1998)

Crystal structures of cofactor-free RTPR from *Lactobacillus leichmannii*, and RTPR bound to adeninylpentylcobalamin, have recently been reported.[113] They show cysteine 408, AdoCbl, and the substrate to all be in locations so that the putative mechanism (Figure 1.12) of RTPR can operate.

RTPR has been called a conundrum because it is a monomeric enzyme with allosteric control.[113,123] The crystal structure shows a 130 amino acid subunit which appears to mimic the interactions seen in dimeric Class I RNRs. Another insight that came from the crystal structure data of RNRs is that Class I RNR from *Escherichia coli* (a non- AdoCbl dependent RNR), Class II RNR from *Lactobacillus leichmannii* (AdoCbl dependent RNR) and Class III RNR (anaerobic bacterial RNR) all have similar active sites in which a radical generation system is in close proximity to a cysteine. This similarity was predicted by Ecklund[124] and by Reichard[125] who believe that all of the RNRs may be part of the same phylogenetic tree. Since the kinetic isotope studies have yielded normal values (1.6 to 2.7),[121] QM tunneling is not suspected in RTPR and, hence, it will not be discussed further.

The first and only crystal structure of the cobalamin-binding domain of a MeCbl dependent enzyme was the 1994 report of methionine synthase (MetH) from *Escherichia coli*.[107] This was the first example showing the 5,6-dimethylbenzimidazole base-off motif with, instead, a histidine imidazole coordinated to the cobalt and the axial base and phosphodiester side chain bound in an enzymatic pocket. MetH catalyses the conversion of homocysteine into methionine with the transfer of methyl cation from MeCbl, leaving Co(I)Cbl as an intermediate. Large conformational changes in the enzyme have been postulated, including a ligand triad of His, Asp, and Ser which control the Co-N bond

length.[126] The cobalamin is re-methylated by the enzyme using methyl-tetrahydrofolate, in a reaction that is not possible to do outside of the enzyme and is, therefore, still under study. MetH is also able to regenerate its cobalamin in the event of a side reaction leading to Co(II)Cbl. These reactions, as well as the role of a zinc binding site and the involvement of Cys-S-Zn species, are still of significant interest. There is no evidence for QM tunneling in this enzyme; hence it will not be discussed further.

The study of AdoCbl itself, and the synthesis and study of analogs of AdoCbl, have proven to be invaluable as points of comparison to the B_{12} enzymes themselves. The initial measurement of Co–C bond strength and the discovery of the 10^{12} enzymatic acceleration of the homolysis of AdoCbl's Co–C bond is a case in point.[51] This dissertation provides a second valuable comparison point by studying QM tunneling in hydrogen atom abstraction by Ado$^{\bullet}$ from substrate and then comparing the results to AdoCbl-dependent enzyme systems. Is there enzyme-enhanced tunneling as Klinman has hypothesized? (This hypothesis will be discussed in more detail later in this chapter.)[127] The study of AdoCbl itself (Chapter 3), as well as the synthesis and study of 8-MeOAdoCbl[128] (chapters 1 and 3), and NpCbl (chapter 4), are all important steps in our understanding of AdoCbl.

HISTORY AND OVERVIEW OF QUANTUM-MECHANICAL TUNNELING

Quantum-mechanical (QM) tunneling is defined as "the finite probability that a particle can pass through a potential energy barrier in quantum mechanics even though a classical particle would be unable to."[129] It has been shown to play a significant role in electron transfer, nuclear decay, and optical phenomena as well as in the transfer of light atoms.

The first example of QM tunneling (although it was not identified at the time) was an optical distortion first reported by Newton[130] in 1704. After the development of quantum mechanics, Newton's observations were termed frustrated total reflection and were understood to be an early example of QM tunneling.[131,132] Theoretical and experimental work on tunneling began with Hund's calculation of the energy levels in a double well potential in 1927.[133] Many other examples of tunneling followed rapidly. Oppenheimer was the first to incorporate QM tunneling into his calculations of the ionization energies of electrons in the hydrogen atom.[134] Nordheim and Fowler demonstrated the first experimental example of an electron tunneling in their "Electron Emission in Intense Electric Fields" work. They measured the velocity of emitted electrons and showed that they did not have sufficient energy to surmount the energy barrier required for their ejection.[135,136] QM tunneling was applied to radioactive disintegration by Gamow,[137] and independently by Gurney and Condon, where QM tunneling was actually a simpler hypothesis than the previous hypothesis of "special arbitrary instability of the nucleus".[138,139] (Although an α-particle has a relatively large mass, it must only move ~ 10^{-14} m for coulombic repulsion to favor its emission. This

very narrow barrier leads to a mechanism of emission which is dominated by QM tunneling (vide infra).)

In 1929, Bourgin derived a barrier penetration term using a simple Schrödinger equation.[140] (The method of using an approximate wave function to determine energy levels in a bound system was originally pioneered by Brillouin, Wentzel, Kramers, and Jeffreys and is called BWK or JWKB approximation.[141-144]) Bell has subsequently calculated a barrier permeability term for barriers of different shapes including: rectangular, parabolic, Eckart, triangular, and inverse Morse potential,[131] using JWKB approximations, as well as exact values for the rectangular and parabolic barriers. The important parameters can be illustrated using the simplest equation for the rectangular barrier (Equation 1.1).[131]

$$G \approx \frac{16W(V_o - W)}{V_o} \exp\left\{-2\left[2m(V_o - W)\right]^{1/2} a/\hbar\right\}$$

Equation 1.1

The equation for barrier permeability for a rectangular symmetrical barrier with relatively low permeability. [131]

G = barrier permeability
W = energy of the tunneling particle
m = mass of the tunneling particle
a = barrier width
V_o = barrier height

From this equation, it can be seen that both the particle mass and the barrier width are in the exponential part of the expression for barrier permeability. This permeability directly relates to tunneling probability and is used to calculate a tunneling correction factor often

abbreviated Q_t (a derivation of Q_t from a barrier permeability is given at the end of this chapter). A qualitatively illustrative example is to use the Heisenberg uncertainty principle to try to understand tunneling. The uncertainty in the position of a particle from the Heisenberg uncertainty principle can be approximated as $\lambda/4\pi$ (λ=the de Broglie wavelength of the particle) (Equation 1.2).

$$\Delta x \approx \frac{\hbar}{2(2mE_k)^{1/2}} \approx \frac{\lambda}{4\pi}$$

Equation 1.2

A qualitative relation of the Heisenburg uncertainty principle to the de Broglie wavelength. [131]

Δx = uncertainty in position
λ = the de Broglie wavelength = $h\ (2mE_k)^{-1/2}$
E_k = kinetic energy

As the mass of a particle decreases, its de Broglie wavelength increases, until the uncertainty in the position of a particle approaches the length of a reaction coordinate. The de Broglie wavelength (λ) for a proton with 20 kJ mol^{-1} of kinetic energy is 0.63 Å,[131] showing that tunneling should begin to play a factor in proton transfer reactions. This example also shows why a deuterium (D) or tritium atom will have a significantly smaller tunneling component in its reactions (with λ = 0.45 and 0.36 Å respectively) than hydrogen. It is this behavior that makes a kinetic isotope effect (KIE) the most valuable tool in the diagnosis and evaluation of QM tunneling.

A ground-state zero-point-energy (GS-ZPE) KIE is caused by a difference in the zero point energy of the R–H or R–D stretching vibration. An equation for the calculation of KIEs neglecting the "tunnel effect" was published in 1949 by Bigeleisen.[145,146] Using a simplified version, (Equation 1.3) a GS-ZPE KIE can be calculated at any temperature and compared to measured values. QM tunneling will cause a significant increase in the KIE (Figure 1.13) which has been used as a valuable in diagnosis of tunneling in many different systems. This tool is limited at low temperatures (< 85 K) where the GS-ZPE KIE has a large value (~ 600 at 85 K) making the reaction rates between hydrogen and deuterium too different to measure under otherwise identical conditions, even in reactions without tunneling.

$$\frac{k_H}{k_D} \approx \exp\left[\frac{hc}{2k_BT}(\nu_{CH} - \nu_{CD})\right]$$

Equation 1.3

The Bigeleisen equation used to calculate GS-ZPE primary kinetic isotope effects which arise only from the difference in zero-point energy of the isotopes under study.[147]

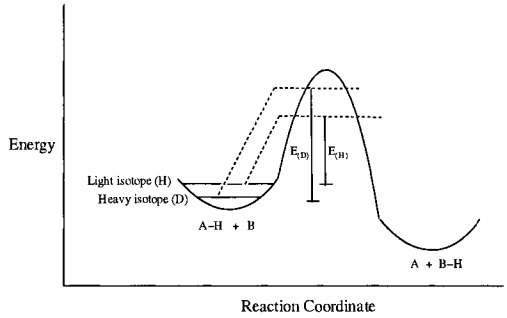


Figure 1.13

An energy diagram illustrating that QM tunneling will give an enlarged KIE as well as a larger value in the differences in activation energy (E_D-E_H) than is expected from non-tunneling behavior.

The ratios of the k_H/k_D, the k_H/k_T, and k_D/k_T are also used as possible diagnostics for QM tunneling. The ratio of these three KIEs can be shown to follow the Swain-Schaad equations (Equation 1.4).[148]

$$k_H/k_T = (k_H/k_D)^{1.442} \qquad k_H/k_D = (k_H/k_T)^{2.26} \qquad k_H/k_T = (k_D/k_T)^{3.26}$$

Equation 1.4

The Swain-Schaad relationships between all three kinetic isotopes of hydrogen.[148]

The idea that QM tunneling would cause a change in these ratios was first reported by Saunders in 1985.[149] He showed that the measured tritium KIE (k_H/k_T) was larger than the value calculated from the deuterium KIE(k_H/k_D) as would be expected from QM tunneling. However, he noted that in cases of moderate tunneling, the expected deviation is only ~ 10%, requiring precise data for interpretation. Recently, Kohen has also cautioned against using the expected relation $(k_H/k_T)=(k_D/k_T)^{3.26}$, and recommended a new limit of $(k_H/k_T)=(k_D/k_T)^{4.8}$ as a maximum non-tunneling limit.[150]

A more commonly used manifestation of QM tunneling is the non-linear Arrhenius behavior in the temperature dependence of a reaction in which tunneling has played a significant component (Figure 1.14).

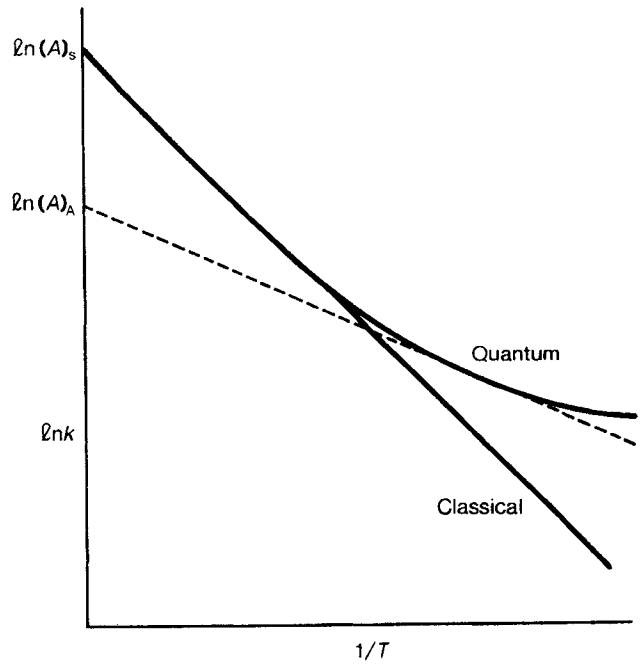


Figure 1.14

A diagram of the Arrhenius behavior that shows the expected curvature, and difference in pre-exponential factor that can be used to diagnose QM tunneling. (Adapted from Bell, R. P. *The Tunnel Effect in Chemistry*; Chapman and Hall: New York, 1980).

From this plot, it is easy to see that at lower temperatures, as the amount of quantum mechanical tunneling becomes larger, the slope of the Arrhenius plot decreases. The Arrhenius pre-exponential factor obtained from an extrapolated intercept will be lower in a case with a significant amount of tunneling. A mathematical derivation of this behavior has been reported by Bell.[131] A comparison of a ln k vs 1/T Arrhenius plots for hydrogen transfer and deuterium transfer can be compared. Because there is more

tunneling in the hydrogen case, its activation energy and pre-exponential factor will both be lowered significantly. A criterion for using this behavior was established by Kwart[151] and also by Kreevoy.[152] Strong indicators of tunneling are a difference in the activation energy (E_D-E_H), calculated from the slope of an Arrhenius plot, that is larger than the expected value of 1.20 kcal mol^{-1} and a ratio of pre-exponential factors (A_H/A_D), calculated from the intercept of an Arrhenius plot, less than 0.7.

Recently, the criteria for QM tunneling has been expanded to include 4 regions of behavior (Figure 1.15).[153,154]

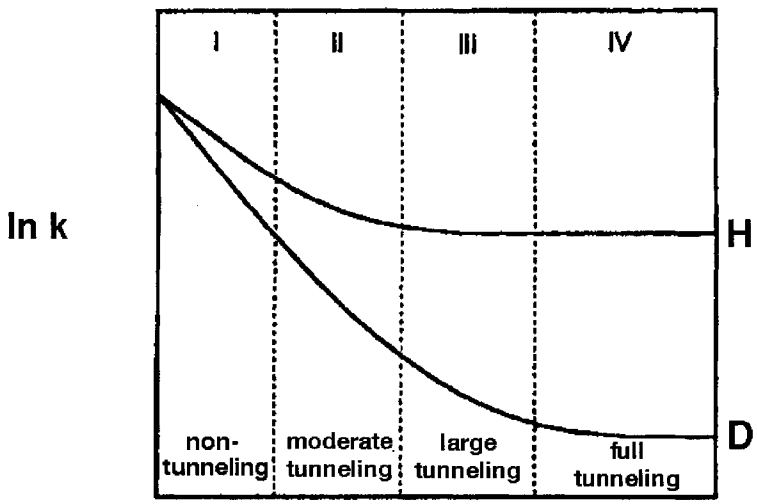


Figure 1.15

The four theoretical regions of QM tunneling behavior. (Adapted from Kohen, A.; Jonsson, T.; Klinman, J. P. *Biochemistry* **1997**, *36*, 6854).

Region I, the non-tunneling region, shows non-tunneling behavior Arrhenius behavior with $A_H/A_D \sim 1$. Region II, the moderate tunneling region, follows the Kreevoy criteria with curved Arrhenius plots and $A_H/A_D < 1$. Region III, the large tunneling region, shows large deviations from Arrhenius behavior, and $A_H/A_D > 1$. Region IV, the full tunneling region, shows no temperature dependence on the rate or on the kinetic isotope effect with $A_h/A_D > 1$. The importance of using more than the A_H/A_D criterion for the diagnosis of tunneling is made obvious by its possible range of values. Criteria for these ranges have been developed by Kuznetson[155]. Many of the examples that follow will be in region II, with a moderate tunneling contribution and, where reported, $A_h/A_D < 1$.

The first evidence of QM tunneling in a hydrogen transfer was reported in 1956 for the bromination of 2-carbethoxycyclopentanone from 10-70 °C by Bell.[131,156] A ratio of pre-exponential factors of $A_H/A_D = 0.04 \pm 0.007$ was observed and explained using a QM tunneling model.

Another early system where QM tunneling is suspected is in the hydrogen atom abstraction from methane by methyl radical at 200-350 °C where a large Arrhenius activation energy difference (E_D-E_H) of ~ 3.5 kcal mol^{-1} was reported.[157,158] (It is of interest here that the Arrhenius activation energy difference in the hydrogen abstraction from ethane by methyl radical only shows E_D-E_H of ~0.6 kcal mol^{-1} at 400 °C.)[159] Many other examples followed including the extensive study of methyl radical abstractions from acetonitrile or methanol glass at low temperatures (5-97 K).[160-162] From these reactions, Siebrand and Ingold devised a new tunneling correction method called the "golden rule treatment of hydrogen transfer reactions".[163-165] This method treats the reactants and products as separate states and describes the rate of transfer with an

interaction operator.[166] This method has the advantage of not separating the tunneling and non-tunneling contributions to the reaction rate, as no barrier ever enters the reaction picture.

Isomerization of aryl radical has also been studied by Ingold.[167-169] The study of the isomerization of 2,4,6-tri-butylphenyl radical (Figure 1.16) has been shown to have a very large KIE (k_H/k_D) of 13000 at 123 K (the GS-ZPE expectation of this KIE at 123K is ~260).

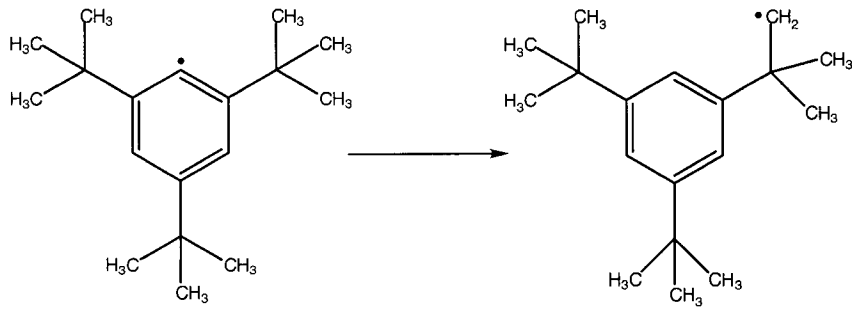


Figure 1.16

The first well studied system involving QM tunneling of hydrogen in an intramolecular hydrogen transfer.[167-169]

Overall, there are 54 different 1-step proton or hydrogen atom transfers where tunneling has been reported,[131,170] and certainly many more will utilize a tunneling mechanism under certain conditions.[131,170] Siebrand believes that tunneling is prevalent enough that we need to change the way that we look at hydrogen transfer reactions, using a method where an energy barrier never enters the reaction picture, "the hydrogen atoms themselves, one presumes, are unaware that they are tunneling... moreover, not all observers will see the barrier but only those that have not yet overcome their classical prejudice."[164]

QUANTUM-MECHANICAL TUNNELING IN ENZYME SYSTEMS

The idea that an enzyme may use QM tunneling in proton transfers was first reported by Gold in 1971.[171] He studied "facilitated" proton transfer reported by Wang[172] in chymotrypsin and carbonic anhydrase. Gold[171] proposed an early version of Klinman's hypothesis that "enzymes may act by forcing small reductions in the lengths of hydrogen bonds, thereby increasing the probability of quantum mechanical tunneling." This work was followed by an early report from Banacky[173] in 1981 that pointed to QM tunneling in the rate determining proton transfer in chymotrypsin.

There were no other reports in the area until 1989 when Klinman obtained the first widely accepted evidence of QM tunneling of hydrogen in the enzyme system Bovine Serum Amine Oxidase.[176] That report was closely followed by her report of hydrogen QM tunneling in Yeast Alcohol Dehydrogenase (YADH).[174,175] There have been many reports since (Table 1.2), with most of the significant work from the labs of Klinman[127,153,174-187] and Sutcliffe.[154,188-193]

Table 1.2

Reports of tunneling in non-AdoCbl dependent enzyme systems (AdoCbl dependent enzymes will be covered in the next section).

Enzyme	Tunneling Evidence	Year	Additional Notes
chymotrypsin	High enzymatic efficiency. Activation energy calculated to be very high (170 kJ mol^{-1}). Close energy match between vibrational energy transfer and leaving-group bond strength.	1968[172] 1971[171] 1981[173]	Proton transfer to a substrate. Normal isotope effect k_H/k_D ~2.2
Carbonic Anhydrase	Proposed conformational change which could decrease proton / substrate distance allowing proton tunneling.	1968[172] 1971[171]	Proton transfer to a substrate. "when proton tunneling is involved, a much more potent catalytic mechanism is available."[171]

Yeast Alcohol Dehydrogenase (YADH)	Significant deviations from the Swain-Schaad relationship. $k_H/k_D = 7.14$ (non-tunneling 3.0) $k_D/k_T = 1.70$ (non-tunneling 1.4)	1989[174]	Hydride transfer from benzyl alcohol to nicotinamide adenine dinucleotide. This was the first widely accepted report of QM tunneling of hydrogen in an enzyme system.
Bovine Serum Amine Oxidase (BSAO)	$A_H/A_D = 0.12$ $A_D/A_T = 0.51$ Large primary k_D/k_T and k_H/k_T	1989[176]	Loss of proton from benzylamine.
Monoamine Oxidase B	Temperature dependence of k_H/k_T and k_D/k_t	1995[177]	Hydrogen atom abstraction. First demonstration with radical intermediates.
Methane Monooxygenase (MMO)	$k_H/k_D = 50\text{-}100$	1996[194]	Hydrogen atom abstraction from methane
Triosephosphate Isomerase	$(k_H/k_T)=(k_D/k_T)^{4.4}$	1996[195] 2002[196]	Proton transfer from substrate to Glu_{165} Potential energy surface calculations suggest only a 5 to 10-fold enhancement of

			tunneling in the enzyme when compared to solution.
Glucose Oxidase	Low enthalpy of activation (8.1 ± 0.4 kcal mol $^{-1}$ in the lowest case) Large (A_D/A_T = 1.47 ± 0.09) (upper limit for non-tunneling values is 1.22)	1997[185] 2002[186]	C–H bond cleavage in glucose. 3 different forms show that remote changes in glycosylation of the surface of the protein affect the tunneling parameters, "less glycosylation results in more tunneling".[185]
Galactose Oxidase	k_H/k_D ~ 22.5 (4° C) k_H/k_D ~ 13 (45 °C)	1998[197]	Proton transfer from a galactose analog to the enzyme.
Themrophylic Alcohol Dehydrogenase (ADH)	A_H/A_T = 0.26 ± 0.23 at 5-30 °C A_H/A_t = 4.3 ± 0.6 at 30-65 °C	1999[180]	The enzyme may be changing from region II to region III behavior
NAD-Malic Enzyme	Swain-Schaad exponent $(k_H/k_T)=(k_D/k_T)^{2.2}$	1999[198]	Hydride transfer from l-malate to nicotinamide
Soybean Lipoxygenase	k_H/k_D ~ 40	1999[179]	Hydrogen abstraction from

1 (SLO-1)	temperature independent	2001[184]	linolic acid.
Hetero tetrameric Sacrosine Oxidase (TSOX)	Kinetic isotope effect studies suggest vibrationally enhanced ground state tunneling (VEGST) is occurring	2000[192]	Proton or hydride transfer from sarcosime to flavin
Δ^9-desaturase	$k_H/k_D = 22.9$ at C9	2000[199]	Hydrogen atom abstraction from 11-tetradecenoic acid $k_H/k_D = 1.0$ at C10.
Horse Liver alcohol dehydrogenase (HLADH)	Temperature dependence the k_H/k_D $A_H/A_D < 0.7$	2000[181] 2001[183]	Some kinetic complexity is observed. Hydride transfer from benzyl alcohol substrate to nicotinamide. Mutations at the active site have little effect on the observed tunneling.
Trimethylamine Dehydrogenase	Temperature independent	2001[190]	Proton abstraction from trimethyl amine by flavin.

(TMADH)	$k_H/k_D = 4.6 \pm 0.4$ $A_H/A_\Delta = 7.8 \pm 1.0$		
Methylamine Dehydrogenase (MADH)	k_H/k_D=11.1 at 273 K	2001[191]	Proton abstraction from methylamine by Asp_{428}. k_H/k_D could be accurately modeled with QM/MM
Peptidylglycine α-Hydrosylating monooxygenase (PHM)	$A_H/A_D = 5.9 \pm 3.2$ E_D-$E_H = 0.37 \pm$ 0.33 kcal mol^{-1} Comparison to SLO-1	2002[187]	Hydrogen atom abstraction from glycine containing peptides. The data could not be fit with a simple tunneling correction. Low activation energy difference
Tyrosine Hydoxylase	$k_H/k_D = 9.6 \pm 0.9$ secondary k_H/k_D = 1.21 ± 0.08	2002[200]	Hydrogen atom abstraction from benzylic substrate

Klinman's work in this area has led her to hypothesize that "the optimization of enzyme catalysis may entail the evolutionary implementation of these chemical strategies that increase the probability of tunneling and thereby accelerate the reaction rate."[177]

There have been studies in an attempt to correlate protein motions or vibrations with an increase in QM tunneling. Schwartz has proposed that a specific internal motion

in the protein may be responsible for QM tunneling in the thermophilic alcohol dehydrogenase system.[201] Sutcliffe has also proposed a vibrationally enhanced ground state tunneling (VEGST) theory, in which the reaction barrier is compressed by the vibrational fluctuations of the protein. Sutcliffe also believes that his theory can be extended to energetically expensive reactions. "In our view, vibrationally enhanced tunneling mechanisms might be more commonplace than currently seen."[189] This theory is supported by calculations from the Hammes-Schiffer group[202] and from the Truhlar[203,204] group for the liver alcohol dehydrogenase system. However, Warshel[205,206] has used computer simulations and found that "vibrationally enhanced tunneling is shown to be a well understood phenomenon that does not lead to special catalytic effects."[205] The Karplus group has also used quantum mechanical / molecular mechanics calculations in an attempt to directly compare the contribution of triosephosphate isomerase in its reactions. They found that only a 5-10 fold increase in the rate could be attributed to an increase in tunneling, which is insignificant compared to the enzymatic acceleration of 10^9.[196]

There is clearly still significant controversy in this area. What is clear is that *any understanding of enzymatic contribution to QM tunneling relies on an accurate estimation of the amount of tunneling in the system without the enzyme*. Hence, this dissertation will provide such a measurement for the first time, using an AdoCbl plus ethylene glycol enzyme-free solution analog of the reaction catalyzed by diol dehydratase.

COENZYME B_{12} SYSTEMS THAT MAY INVOLVE QUANTUM-MECHANICAL TUNNELING

There has been a report of QM tunneling in the recent AdoCbl dependent enzyme literature. Banerjee has reported QM tunneling in Methylmalonyl CoA Mutase (MMCoA),[207,208] and the experimental results have been supported by calculations[209] which included a zero-curvature tunneling correction (ZCT; a numerical method used to calculate the tunneling or non-classical reflection of particles interacting with energy barriers).[210]

There is also evidence for QM tunneling in other AdoCbl dependent enzymes including the well studied systems of Diol Dehydratase, Ethanolamine Ammonia Lyase,[43,45] and Glutamate Mutase.[40,41,120,211,212] The evidence for this tunneling (Table 1.3) is the same as the evidence commonly used to distinguish tunneling in region II of the 4 region system (vide supra): large KIEs ($k_H/k_D > 6.4$ at 20 °C), large activation energy differences ($E_D-E_H > 1.2$ kcal mol^{-1}), and small ratios of pre-exponential factor ratios ($A_H/A_D < 0.7$). As can easily be seen from the data, the criteria that are available currently point to a QM tunneling contribution in these well-studied reactions. Further discussion of the evidence for tunneling is presented in chapter 3 of this dissertation.

Table 1.3

Evidence for QM tunneling in AdoCbl dependent enzymes.

	KIE	E_D-E_H (kcal mol^{-1})	A_H/A_D	Notes/References
Methylmalonyl-CoA mutase	35.6 at 20 °C 43.1 at 10 °C	3.41±0.07	0.078±0.009	207-209
Ethanolamine ammonia lyase		3.1 ± 1.1	0.038 ± 2.13	43,45
Glutamate mutase	28 to 35 at 10 °C			40,120 $k_H/k_T > 100$ 211
Diol dehydratase	8 for the overall reaction at 10°C 28.6 at 10 °C for hydrogen abstraction step			31,117

WHY OUR STUDIES ARE IMPORTANT

In order to test Klinman's hypothesis that, "The optimization of enzyme catalysis may entail the evolutionary implementation of these chemical strategies that increase the probability of tunneling and thereby accelerate the reaction rate",[177] a comparison of tunneling with and without the enzyme must be made (Figure 1.17).

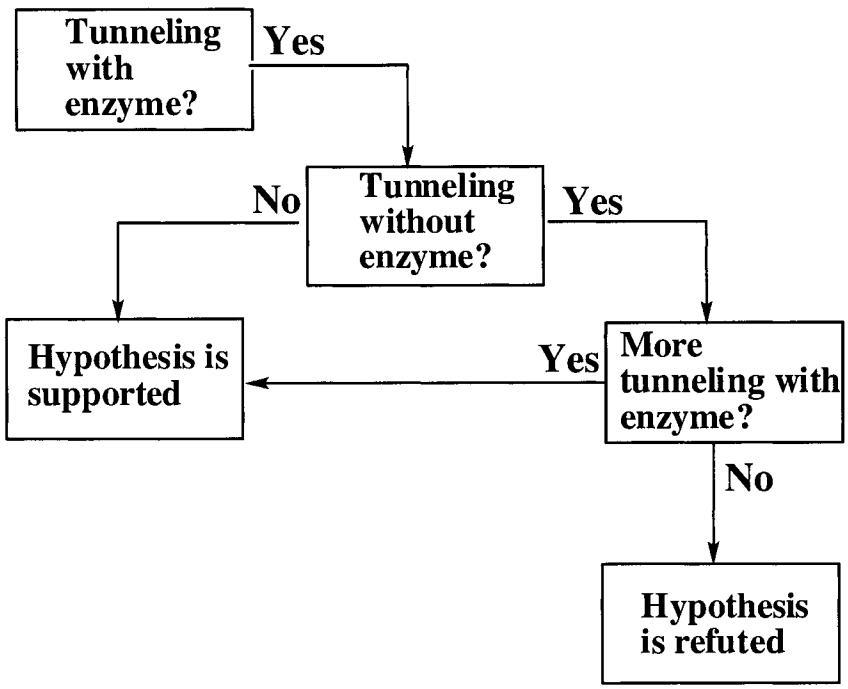


Figure 1.17

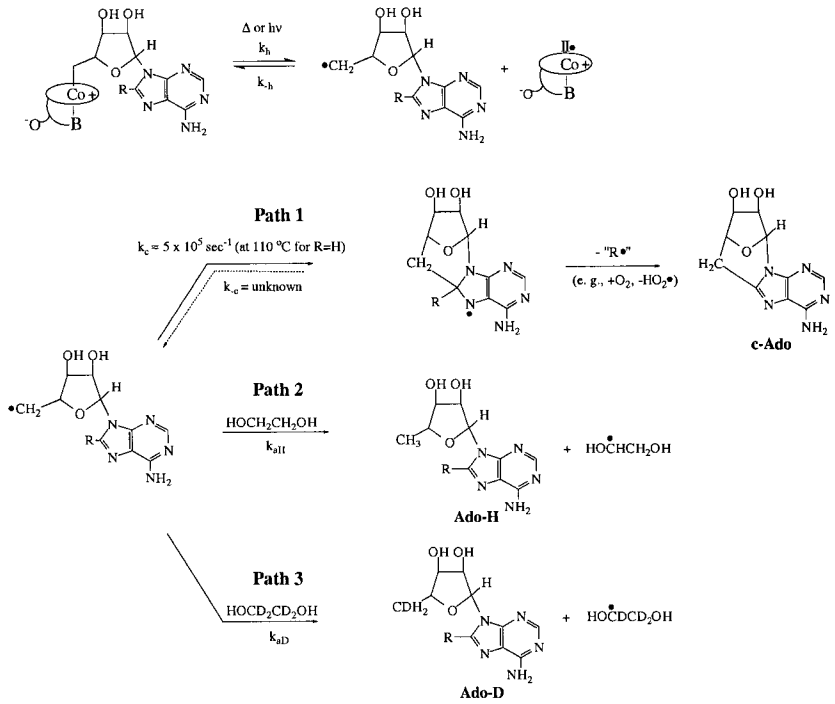
A simple procedure designed to test Klinman's hypothesis.

Klinman,too has expressed the need to find reactions to test her hypothesis asking "Is hydrogen tunneling under physiological conditions a unique feature of biocatalysis, or is it of similar importance in noncatalyzed reactions? This question is still a controversial

one, *as good noncatalyzed reference reactions are hard to find.*"[153] Put another way by Karplus, in reference to the triosephosphate isomerase system (TIM), "that tunneling exists in TIM does not by itself show that it has a role in catalysis. For that one has to compare the tunneling in the enzyme with that in solution."[196]

Despite their importance, there have only been two reported attempts to carry out such a comparison *using calculations*; no experimental systems have been reported. In a calculation regarding the MMCoA system, Paneth was able to show that Arg 207 hydrogen bonded to the substrate was necessary to reproduce experimental results. [209] The other report from the Karplus group used MM/QM methods in the triosephosphate isomerase system. Their results show no significant increase in tunneling in models of solution, gas, or enzyme reactions.

Our study is the first experimental case where the needed enzyme-free to enzyme comparison can be accomplished. This is only possible because the nature of the AdoCbl system allows the generation of the active radical species and the first step of the enzymatic cycle in enzyme-free solution (Figure 1.5)



(The earlier Figure 1.5, reprinted here for convenience)

The reaction of AdoCbl in ethylene glycol solution. The Co–C bond cleavage step and the path 2 H-abstraction reaction are identical to the Diol Dehydratase reaction.

Table 1.4

A table showing the data (indicated by "?") needed to test Klinman's hypothesis.

	KIE	E_D-E_H (kcal mol^{-1})	A_H/A_D
comparable solution reaction	?	?	?
methylmalonyl-CoA mutase	35.6 at 20 °C 43.1 at 10 °C	3.41±0.07	0.078±0.009
ethanolamine ammonia lyase	NA	3.1 ± 1.1	0.038 ± 2.13
glutamate mutase	28 to 35 at 10 °C	NA	NA
diol dehydratase	8 and 28.6 at 10 °C	NA	NA
ground-state ZPE	6.4 at 20 °C 6.8 at 10 °C	1.2	1.0

It is the goal of this dissertation to fill in the question marks in this table. Doing so will allow the first experimental conclusion as to whether or not enzymes enhance QM tunneling in at least AdoCbl dependent H-abstraction reactions.

DERIVATION OF THE TUNNELING CORRECTION TERM, Q_t

(Adapted from Bell, R. P. *The Tunnel Effect in Chemistry*; Chapman and Hall: New York, 1980.)

Using a standard Boltzman energy distribution:

W = the energy of a particle

J = rate at which particles which appear on the right side of the barrier

J_o = total flux of particles reaching the left side of the barrier

G(W) = a barrier permeability

Q_t = the ratio of the quantum-mechanical rate to the non-tunneling rate

$$J = \frac{J_o}{k_B T}\int_0^\infty G(W)e^{\frac{-W}{k_B T}}dW$$

J_c = the rate with no contribution from tunneling

For a barrier height = V_o,

G(W) = 1 for $W > V_o$, G(W) = 0 for $W < V_o$

and J_c can be expressed as:

$$J_c = \frac{J_o}{k_B T}\int_{V_o}^\infty (1)e^{\frac{-W}{k_B T}}dW = J_o e^{\frac{-V_o}{k_B T}}$$

Q_t = the tunneling correction term = J/J_c

$$Q_t = \frac{J}{J_c} = \frac{\frac{J_o}{k_B T}\int_0^\infty G(W)e^{\frac{-W}{k_B T}}dW}{J_o e^{\frac{-V_o}{k_B T}}} = \frac{e^{\frac{V_o}{k_B T}}}{k_B T}\int_0^\infty G(W)e^{\frac{-W}{k_B T}}dW$$

Qualitatively, Q_t will have a minimum value of 1 when there is no tunneling. Bell has defined three regions for Q_t behavior.

Negligible tunneling: $1 < Q_t < 1.1$; tunneling will rarely be detectable experimentally

Moderate tunneling: $1.1 < Q_t < 4$; the range most commonly seen in chemical reactions at ordinary temperatures.

Large tunneling: $Q_t > 4$; will rarely be encountered at ordinary temperatures.

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CHAPTER 2.

The synthesis and characterization of 8-Methoxy-5'-deoxyadenosylcobalamin: a coenzyme B_{12} analog which, following Co-C bond homolysis, avoids cyclization of the 8-Methoxy-5'-deoxyadenosyl radical.

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"Reprinted from Journal of Inorganic Biochemistry, Vol 91, Kenneth M. Doll, Paul E. Fleming, Richard G. Finke, The synthesis and characterization of 8-methoxy-5'-deoxyadenosylcobalamin: a coenzyme B_{12} analog which, following Co-C bond homolysis, avoids cyclization of the 8-methoxy-5'-deoxyadenosyl radical, Pages 388-397, Copyright (2002), with permission from Elsevier Science".

This chapter was reprinted directly as it appears in J. Inorg. Biochem. 91 (2002) 387 with the exception of margins and figure numbering which have been redone in order to comply with the Colorado State University Thesis Manual. This chapter contains a detailed synthesis and characterization of 8-Methoxy-5'deoxy-adenosylcobalamin. It is the first report that contains sufficient experimental details for a reproducible synthesis, plus adequate characterization of this molecule. There is also the first reported demonstration of the homolysis of this molecule into 8-MeOAdo$^{\bullet}$ and Co(II)Cbl$^{\bullet}$ at a rate similar to AdoCbl.

Obtaining this molecule in high purity proved to be very valuable in our studies, as it gave us a direct comparison to our AdoCbl system (more discussion is presented in chapter 3 of this dissertation). This comparison was important in our ability to test Klinman's hypothesis that enzymes have evolved in order to enhance QM tunneling.

Abstract

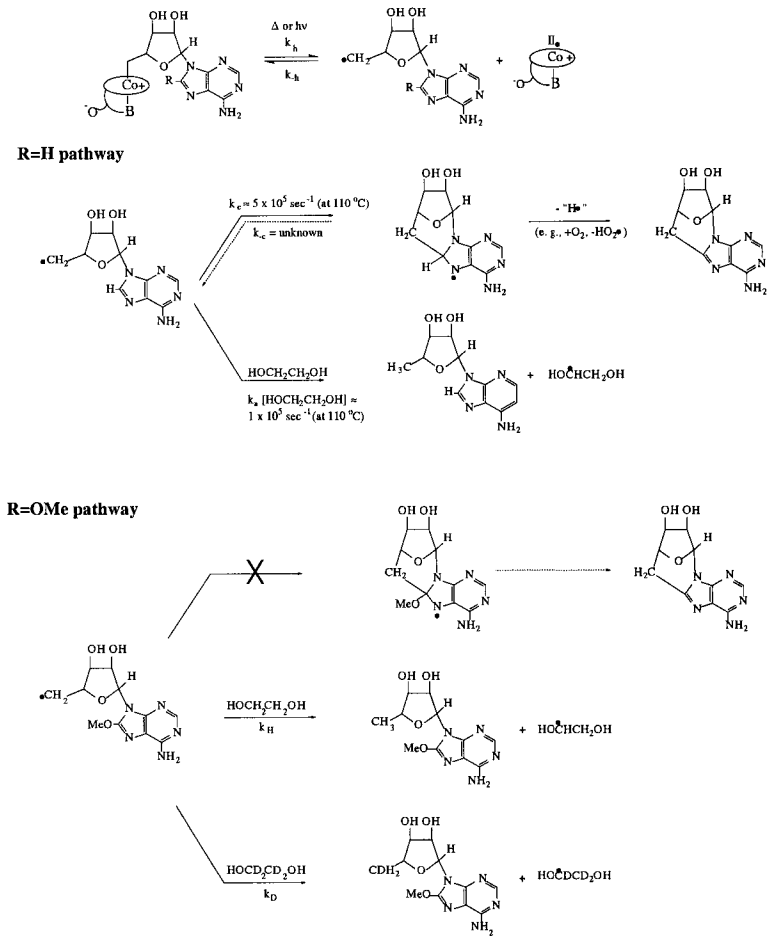
The compound 8-methoxy-5'-deoxyadenosylcobalamin (8-MeOAdoCbl), has been synthesized in 37% yield and purity ≥95% by HPLC, monitored at both 254 nm and 525 nm, or 90 ± 2% purity as judged by the ^{1}H NMR spectrum of the aromatic cobalamin region. This is the first synthesis of this complex in which sufficient details are reported, where a yield and purity are reported, and where key problems in the synthesis and purification are overcome, so that 8-MeOAdoCbl can actually be obtained for use in other studies. Also demonstrated is the clean Co-C bond homolysis of 8-MeOAdoCbl to give initially 8-MeOAdoCbl$^{\bullet}$ and Co(II)Cbl$^{\bullet}$ in a UV-visible thermolysis experiment at 110 °C, which show that the 8-MeO moiety suppresses the cyclization to 8,5'-anhydro-adenosine otherwise seen for the adenosyl radical (Ado$^{\bullet}$). Suppression of this cyclization pathway makes 8-MeOAdoCbl invaluable for studying the kinetic isotope effect (KIE) of the Ado$^{\bullet}$ plus substrate H$^{\bullet}$ abstraction, a component of the first definitive test of Klinman's hypothesis that the optimization of enzyme catalysis may entail strategies that increase the probability of tunneling and thereby accelerate H$^{\bullet}$ atom abstraction reaction rates.

1. Introduction

Coenzyme B_{12} (Adocobalamin; AdoCbl) is an essential cofactor in at least 12 different enzymatic reactions [1]. The first step in B_{12}-dependent rearrangements is widely accepted to be Co-C bond homolysis to give cobalt(II)cobalamin (Co(II)Cbl) and 5'-deoxyadenosyl radical (Ado$^{\bullet}$), a step which has been shown to occur even outside the enzyme [2], albeit at 10^{10}-10^{12} slower rates. The next formal step of the enzymatic cycle is the abstraction of a hydrogen atom from the substrate (R–H) by the Ado$^{\bullet}$ to give 5'-deoxyadenosine (Ado–H) and a substrate radical, although there is growing evidence that the Co-C cleavage and hydrogen atom abstraction steps are at least coupled if not concerted [3-5].

The isotope effect in the Ado$^{\bullet}$ plus substrate H$^{\bullet}$ abstraction is of fundamental interest. First, isotope effects in enzyme catalyzed reactions are difficult to measure and are poorly understood [6,7]; hence, studies of the ostensibly same reactions outside the enzyme are both rare and of significant interest [7]. Second, in preliminary work modeling B_{12}-dependent diol dehydratase, we have measured a kinetic isotope effect (KIE) for the abstraction of a hydrogen atom from the diol dehydratase substrate, ethylene glycol, by an Ado$^{\bullet}$ generated from AdoCbl Co–C bond homolysis. The Ado$^{\bullet}$ cyclization reaction (Scheme 2.1; R=H pathway) and resultant ratio of Ado-H and 8,5'-anhydroadenosine (cyclic-Ado) was used to compare the rate of H and D abstraction. The results revealed a KIE of 12.4 ± 1.1 at 80°C as well as an apparent activation energy difference of 3.0 ± 0.3 kcal mol^{-1} between hydrogen abstraction from ethylene glycol and deuterium abstraction from d_4-ethylene glycol [K. Doll, B. Bender, R. Finke, manuscript

in preparation]. These results are indicative of quantum mechanical tunneling [8,9]. However, the Ado$^{\bullet}$ to cyclic-Ado reaction must be irreversible for the observed kinetic isotope effect to be unambiguously interpreted. Reversibility in this step leads to a situation involving a "competitive reaction isotope effect" with its less straightforward interpretation [10]. What is required then, is an adenosylcobalamin derivative which will prevent the Ado$^{\bullet}$ cyclization reaction thereby allowing the direct observation of the isotope-sensitive abstraction reaction (Scheme 2.1; R=MeO).



Scheme 2.1. The established mechanism of the thermolysis of adenosylcobalamin in ethylene glycol [2,34], (R=H pathway) and the proposed thermolysis scheme for 8-MeOAdoCbl in ethylene glycol (R=OMe pathway) showing how the lack of cyclization of the 8-MeOAdo• will allow a direct measurement of the k_H/k_D kinetic isotope effect of the H• abstraction reaction from the $HOCH_2CH_2OH$ or $HOCD_2CD_2OH$ substrate.

Earlier work indicates that the use of the 8-methoxy-5'-deoxyadenosylcobalamin (8-MeOAdoCbl; Figure 2.1) derivative of adenosylcobalamin suppresses the cyclization reaction [11,12]. Unfortunately, no detailed synthesis, characterization, or purification of 8-MeOAdoCbl is available so that 8-MeOAdoCbl is unavailable by the prior report (a short communication [11]).[1] Indeed, even with the prior work, plus the useful literature on the syntheses of adenosylcobalamin analogues, [13-21], it has taken more than a year of effort by two of us to obtain the satisfactory 8-MeOAdoCbl described in the present work.

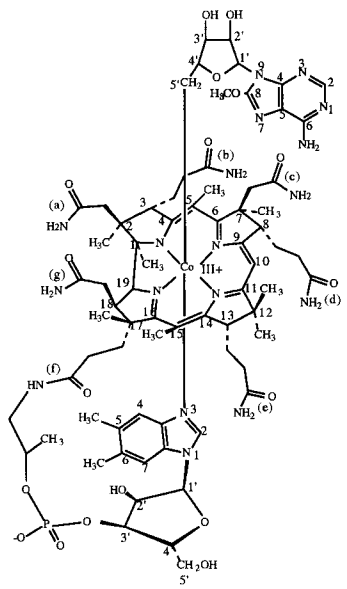


Figure 2.1. The Structure of 8-methoxy-5'deoxy-adenosylcobalamin including the IUPAC numbering scheme.

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[1] The earlier report [11] is a communication void of any experimental details, nor even the resultant yield; satisfactory characterization of the 8-MeOAdoCbl product is missing as well. Moreover, the results herein show that the synthesis, purification, and determination of the purity of 8-MeOAdoCbl are each non-trivial issues.

Herein, we report: (1) the use of a primary alcohol selective tosylation route in the synthesis of 8-MeOAdoCbl;[2] (2) the synthesis of 8-MeOAdoCbl in 37 % yield with a purity of ≥ 95% by HPLC and 90 ± 2% by ^{1}H NMR; (3) characterization of the 8-MeOAdoCbl by ^{1}H NMR, ^{13}C NMR, HPLC, UV-visible spectroscopy, LSIMS (liquid secondary ionization mass spectroscopy), and elemental analysis; and (4) UV-visible spectroscopy evidence for clean homolysis of 8-MeOAdoCbl to Co(II)Cbl and 8-MeOAdo$^{\bullet}$, with elimination of the cyclization pathway seen for Ado$^{\bullet}$. In work in progress, we are using 8-MeOAdoCbl to provide evidence for or against H–tunneling in the Ado$^{\bullet}$ plus R–H to Ado–H plus R$^{\bullet}$ hydrogen abstraction reaction. The present synthetic work is, of course, the key underpinning to that study.

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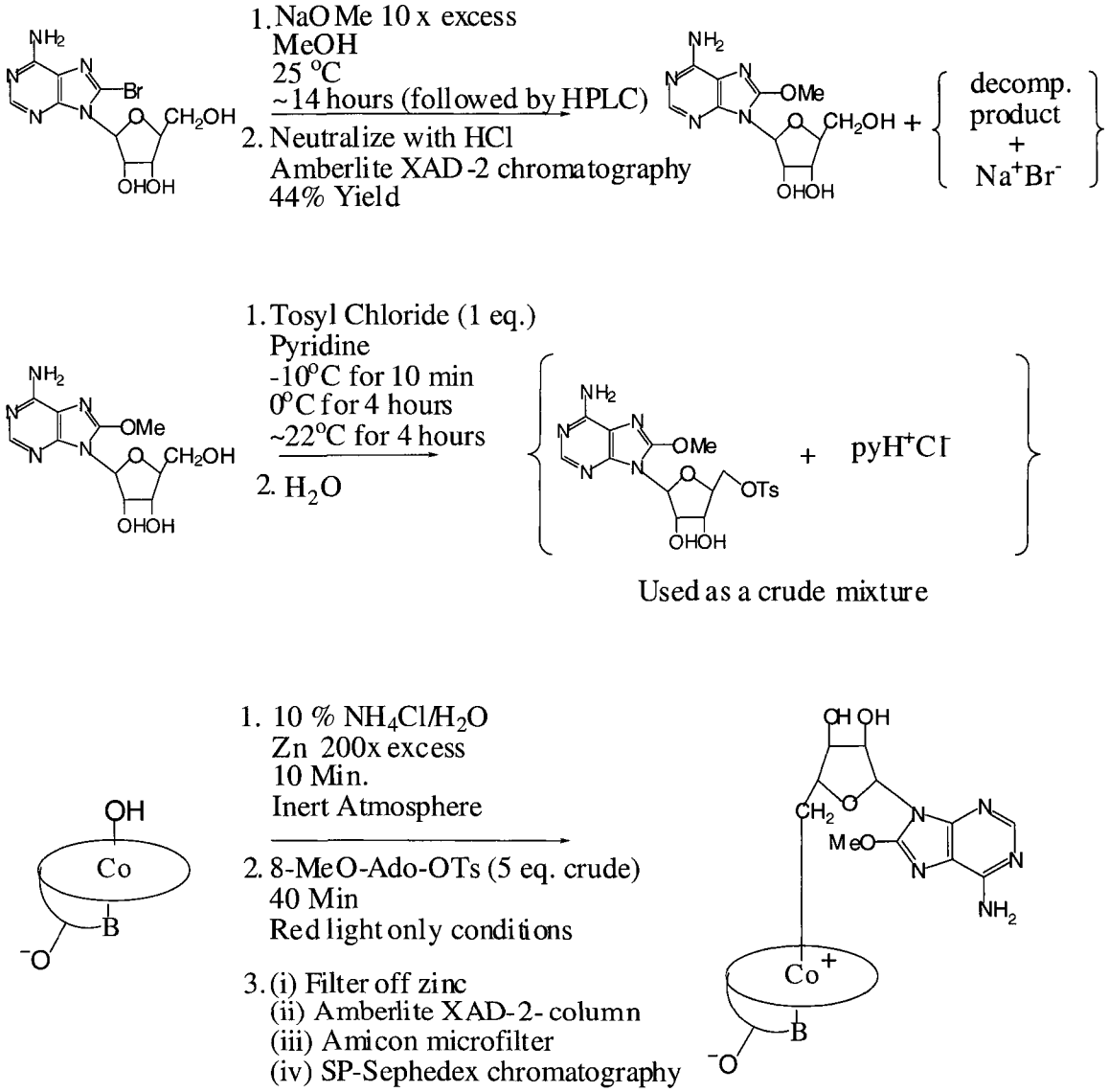
[2] Some literature indicates that it is possible to selectively tosylate the primary Ado-5'-hydroxyl group [17,18] thereby avoiding the need for protection and deprotection. However, the reported yields are low and of questionable precision (ca. 40-50% for adenosylcobalamin, and ca 30% for the 2'-deoxy analogue, by densitometric analysis of paper chromatograms). The low yields have been attributed to the facile cyclization between the 5' carbon and N^3 of the unprotected tosylated nucleoside [19]. Hence, this Ado 5' to N^3 cyclization is a side reaction that must be (and has been) avoided as part of the present work.

2. Results and Discussion

2.1. The Synthesis of 8-MeOAdoCbl

The steps in the synthetic route to 8-MeOAdoCbl are summarized in Scheme 2.2.



Scheme 2.2. The synthesis of 8-MeOAdoCbl developed as part of the present studies.

8-Bromoadenosine can be purchased from Aldrich and shown to have ~99% purity by ^{1}H NMR. We also made 8-bromoadenosine by the literature procedure [22] before it was commercially available (see the Supplementary Material, Section S-1, for details; 71% yield, 98% purity in our hands: literature yield 74% [22]).

The addition of methoxide to 8-bromoadenosine (Section 4.5) was done following the literature of Holmes and Robins [23]. We found, however, that room temperature (~22°C) proved sufficient for the reaction vs the refluxing methanol in the literature [23]. By following the formation of product and the disappearance of starting material by HPLC, we observed a decomposition product late in the reaction. Nevertheless, it is important to allow all of the starting material to react since the separation of the starting material from product proved to be problematic. The 8-methoxyadenosine was purified by chromatography on an Amberlite XAD-2 column eluted with water (96% pure product by HPLC and ^{1}H NMR in 36% yield; literature 44% yield; purity not reported).

The semi-selective[3] [24] tosylation of 8-methoxyadenosine's primary alcohol was accomplished by reaction with one equivalent of tosyl chloride at low temperature (-10 °C; Section 4.6). This tosylation route proved effective, even though it is not 100% selective for the primary alcohol, eliminating the need to protect and deprotect the nucleoside or to use toxic HMPT as done in previous work[4] [25].

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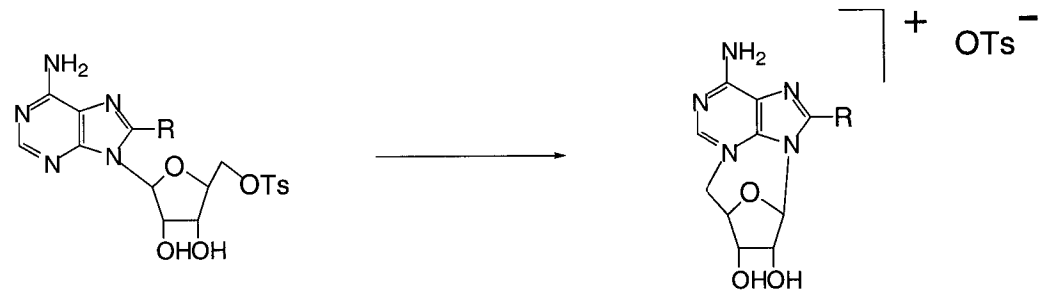
[3] The selectivity for tosylation of the 5'-OH of 8-MeOAdo is probably less than the selectivity in for 5'-OH tosylation in adenosine. This may be due to the preference of a syn conformation in the substituted case[24], allowing the 5'-OH of the 8-MeOAdo to hydrogen bond to the N^3 position. Unsubstituted adenosine prefers an anti conformation disfavoring this hydrogen bonding and increasing relative selectivity for the 5'-OH of simple adenosine.

[4]Bromination of the adenosyl secondary alcohols by the HMPT/$SOBr_2$ approach is expensive, dangerous (due to the high toxicity of HMPT) and not particularly effective

A key question in developing the best synthesis was whether or not to try to isolate and purity the 8-methoxy-5'-tosyladenosine (8-MeOAdoOTs) intermediate, especially in light of the literature indicating there is a facile N^3-5' cyclization reaction (Equation 2.1) [26].



Equation 2.1 The cyclization reaction of the 8-MeOAdoOts intermediate.

In a control experiment, we tosylated simple adenosine in the same procedure used to tosylate 8-MeOAdo–OH, and (vide supra) and then tried to isolate and crystallize the tosylated product (by precipitation from acetone and recrystallization from acetone/water). This attempt produced crystals with a ^{1}H NMR similar to the cyclization decomposition product, N^3-5'-cycloadenosine [27] (Supplementary Material,

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for the target compound. Using the HMPT/$SOBr_2$ method, our yields of this compound were <10% impure product. Similarly, chlorination via the $SOCl_2$ approach failed completely in our hands, despite a control experiment demonstrating our successful use of the $SOCl_2$ route in the synthesis of other cobalamin analogs [25].

Section S-3) indicating that it was necessary to use the 8-MeOAdoOTs as prepared and as earlier AdoCbl analog syntheses had done [16,18].[5]

Oxidative addition of the freshly prepared 8-MeOAdoOTs (5 equivalents) to the Co(I)Cbl supernucleophile proceed as expected. Co(I)Cbl was generated in an inert atmosphere glovebox by the standard method of reduction of HOCbl with zinc in a 10% NH_4Cl aqueous solution [28]. A dark red to green-black and then to bright red color change signified the HOCo(III)Cbl to Co(I)Cbl to 8-MeOAdoCbl reaction sequence.

2.2. Purification of 8-methoxyadenosylcobalamin

The purification of 8-MeOAdoCbl employed a desalting step using an Amberlite XAD-2 column followed by the use of an Amicon YC-05 membrane ultrafilter with a molecular weight cutoff of ca. 500 Daltons. The desired cobalamins are retained on the

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[5] We also performed control experiments (Supplementary Material Section-S4) to test the rate of cyclization in a methanol/water solution. Those experiments show that the decomposition half-lives of both 5'-tosyladenosine (5.8 ± 0.2 days) and 5'-chloroadenosine (≥ 190 days) are much greater than the required reaction time for tosylation and addition to the cobalamin in our synthesis (~18 hours for the reactions and the first purification column). This indicated that we could use the tosylated compound without isolation, and without losing a significant amount due to cyclization. However, analogues may cyclize more readily due to restrictions of conformational freedom and electronic effects caused by the substituent. For example, cyclization of 8-methoxy-5'-tosyladenosine, our starting material, has been reported to be faster than that of the parent 5'-tosyladenosine [19] for two reasons: (1) the electron donating resonance effect of the methoxy group increases the electron density on N^3 and hence, its nucleophilicity; and (2) conformational changes [24] caused by the methoxy group may increase the rate of cyclization. Cyclization can be especially problematic if one needs to use the nucleoside starting material as the limiting reagent. Hence, in the synthesis described herein, the cobalamin starting material has been used as the limiting reagent and the nucleoside has been used in excess to minimize this problem. This mandates the use of an ultrafiltration membrane in the purification step to separate the larger 8-MeOAdoCbl from the excess 8-MeOAdoOTs.

membrane while low molecular weight contaminants pass through it.[6] This procedure yielded ~80% pure product (by HPLC), hence further purification proved necessary[7] and was achieved using a Sephadex-SP C-25 column. The product containing fractions were lyophilized giving a final isolated yield of 8-MeOAdoCbl of 37% with 95% purity by HPLC and 90 ± 2% cobalamin purity by the aromatic region of its ^{1}H NMR spectrum (Section 2.3). Crystallization was investigated, but proved untenable, for further purification of the somewhat sensitive 8-MeOAdoCbl.[8]

2.3. Determination of the purity of 8-methoxyadenosyl cobalamin

Our methods of purity detection[9] emphasize HPLC, with detector wavelengths of 254 nm where possible nucleoside impurities have extinction coefficients comparable to

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[6] Ideally, the filter would, in one step, desalt and remove low molecular weight components from the reaction mixture. In practice we found that the filters worked more efficiently if desalting was carried out using an XAD-2 column prior to filtration. Otherwise filtration rates were unacceptably slow, on the order of days rather than the hours otherwise required.

[7] The 20% impurities, which can be removed with ion exchange chromatography, include HOCo(III)Cbl and what has been tentatively identified (vide infra) as the overtosylated 8-MeOAdoCbl product. The main impurity, which eluted ahead of the desired product, exhibited a mass spectrum with a molecular ion peak at m/e 1747.5. This corresponds to an 8-methoxy-5'-deoxyadenosylcobalamin in which either the 2' or the 3' hydroxyl group has been tosylated (calculated 8-methoxy-5'-deoxy-X'-OTs-adenosylcobalamin $+H^+$ m/e = 1747.7), indicating that the tosylation of 8-MeOAdoOH at its primary alcohol, as expected, is not completely selective.

[8] Further purification through recrystallization from water acetone solution was considered, but then ruled out by control experiments using AdoCbl. First, the recovery of AdoCbl from the recrystallizations was only 44-61%. In addition, the purity of the recovered material was *lower* (by HPLC) than that of the starting material, strongly suggesting that recrystallization is not a viable option for purification of 8-MeOAdoCbl This result is not unexpected: crystallization is rarely used as a purification method in the literature of alkylcobalamin syntheses.

[9] Historically, cobalamins have often been characterized using only UV-visible spectroscopy and paper (or thin layer) chromatography. As others [16] have also noted,

cobalamins [29], and ^{1}H NMR of the aromatic region, which has recently been shown to be the most effective means of detecting cobalamin impurities that otherwise are often missed [30]. LSIMS (liquid secondary ionization mass spectroscopy), UV-visible spectroscopy, and elemental analysis were then used to confirm the product composition and purity.

HPLC shows ≥95% purity using an isocratic 85/15% H_2O/CH_3CN with a flow rate of 2 mL/min on a 300 mm C-18 reverse phase column monitored at both 525 nm and 254 nm. As expected, the UV-visible spectrum (Supplementary Material, Figure 2.S1) of 8-methoxyadenosylcobalamin is very similar to that of adenosyl cobalamin [29].

The ^{1}H NMR spectrum of 8-MeOAdoCbl (Figure 2.2) is very similar to that of AdoCbl [31,32], except that the AdoCbl resonance at δ 8.00 assigned in the literature to 8-H is missing, as expected due to the 8-MeO substitution, and the AdoCbl resonance at δ 5.56, assigned to the proton at the A1' position, is shifted slightly to δ 5.45 for 8-MeOAdoCbl. The ^{1}H NMR spectrum shows the material to be 90 ± 2% pure in the aromatic cobalamin region.

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these methods are inadequate, especially for detecting small amounts of nucleoside contaminants.

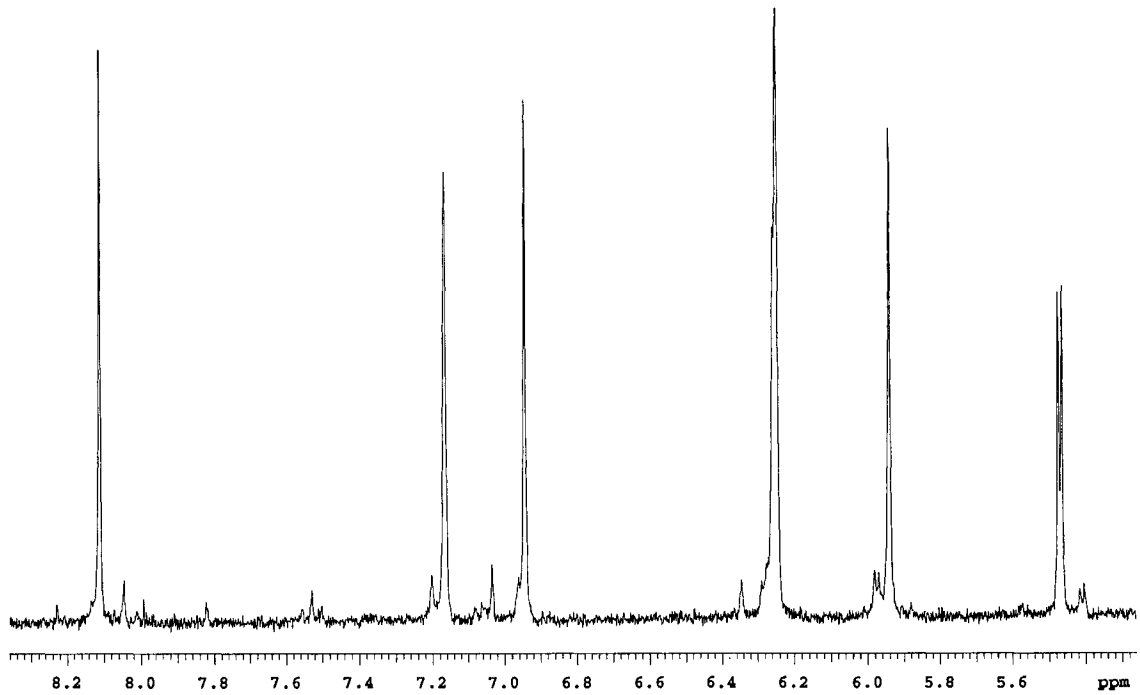

Figure 2.2. The ^{1}H NMR of 8-MeOAdoCbl (D_2O referenced to TSP) δ 8.11(s, 1H) δ 7.17 (s, 1H) δ 6.95 (s, 1H) δ 6.25(d, 2H) δ 5.95 (s, 1H) δ 5.45(s, 1H). Purity is ~ 90% by this ^{1}H NMR.

The natural abundance ^{13}C NMR of 8-MeOAdoCbl was also taken (Supplementary Material, 2.S2). The spectrum is consistent with the completely assigned spectrum of AdoCbl [31]. As expected, there is an additional peak at 60.6 ppm, the correct position for the methoxy carbon [33]. The spectrum also shows the A8 peak shifted by ~10 ppm to 154.1 and the A1' peak was shifted upfield by ~0.8 ppm to 90.2 ppm.

LSIMS (Supplementary Material, Figure 2.S3) reveal an m/e of ($M+H^+$)=1610.2. This corresponds to the anticipated molecular formula for 8-MeOAdoCbl of $C_{73}H_{102}N_{18}O_{18}PCo{\cdot}H^+$ (calculated m/e=1609.7). Other fragments are those expected [34] including 1329.5 ($M+H^+$–8-methoxyadenosyl group), 1069.5 ($M+H^+$–dimethylbenzimidazole-sugar), and 971.5 ($M+H^+$–dimethylbenzimidazole-sugar-phospahate-water).

2.4. Demonstration of Clean Co–C Bond Homolysis of 8-MeOAdoCbl by UV-visible spectroscopy

A sample of 8-methoxyadenosylcobalamin was thermolized at 110 °C for 21 hours (Section 4.10) under anaerobic conditions. UV-visible spectra were taken at regular intervals (Figure 2.5) showing conversion of 8-MeOAdoCbl to Co(II) with clean isosbestic points at 337, 392, 486, and 585nm, characteristic of the conversion of an adenosylcobalamin to its corresponding Co(II)Cbl species [35]. Linear plots of $\ln[(A_0-A_\infty)/(A-A_\infty)]$ vs. time (Supplementary Material, Figures 2.S4 and 2.S5), monitoring the disappearance of the 376 nm peak and the appearance of the 474 nm peak, have a slope corresponding to a first-order rate constant of 8.5 (± 0.2) x 10^{-5} s^{-1}. This is similar to the 110 °C thermolysis rate constant for adenosylcobalamin (11 x 10^{-5} s^{-1}, corrected for radical trap contribution) [35], results which show that the 8-MeO substitution has, as desired, a negligible effect on the rate of Co-C bond homolysis. HPLC on the resulting solution shows the absence of the cyclic-Ado peak indicating that the cyclization reaction has been avoided.

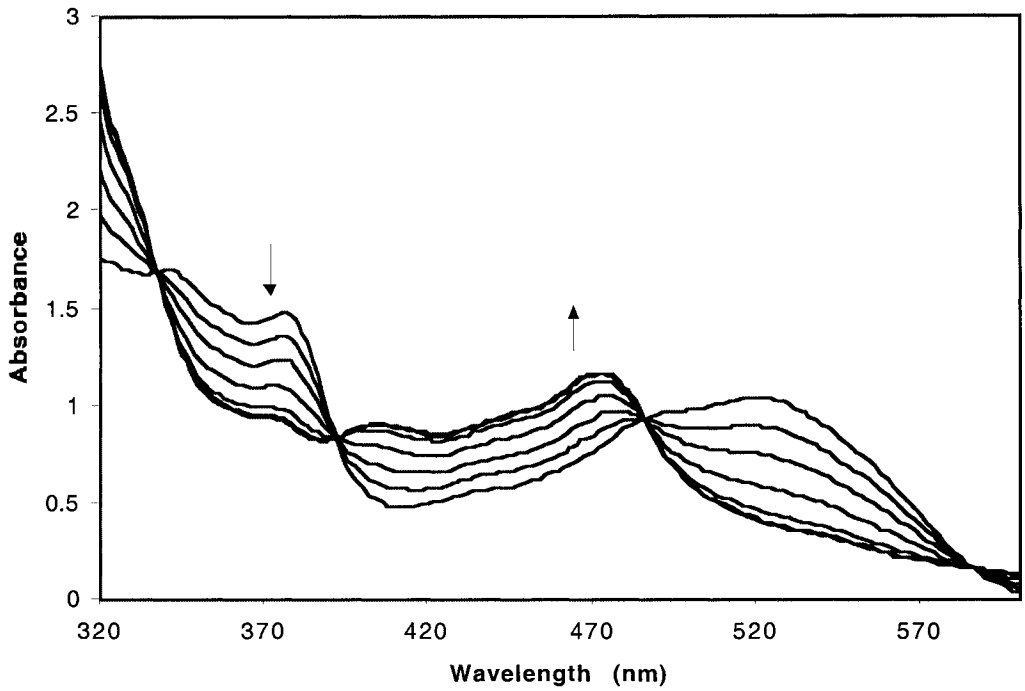

Figure 2.3. The UV-visible spectra of 8-MeOAdoCbl in anaerobic ethylene glycol at 110 °C at 0, 45, 101, 202, 404, 704, 1160, and 1260 min. Clean isosbestic points are observed at 337, 392, 486, and 585 nm indicating quantitative conversion to Co(II)Cbl and 8-MeOAdo•.

3. Summary

An effective synthesis for 8-methoxy-5'deoxy-adenosylcobalamin has been developed, the first synthesis which allowed this useful complex to actually be physically obtained and in known purity. We have also demonstrated that 8-MeOAdoCbl thermolyzes cleanly under anaerobic conditions to give 8-MeOAdo$^{\bullet}$ plus Co(II)Cbl with avoidance of the cyclization reaction seen for Ado$^{\bullet}$. Significantly, the use of the now available 8-MeOAdoCbl is proving most interesting in suggesting hydrogen-atom tunneling in even the enzyme-free chemical reactions of 8-MeOAdoCbl produced 8-MeOAdo$^{\bullet}$ in solution [K. Doll, R. Finke, submitted for publication].

4. Experimental

4.1. Materials

The following were used as received: Adenosine (Sigma), 8-bromoadenosine (Aldrich, 98%), 5'-tosyladenosine (Aldrich) (used to determine rate of intramolecular cyclization), bromine (Baker Analyzed), sodium methoxide (Aldrich, 95%), sodium acetate (EM Science), acetic acid (Baker Analyzed), methanol (Fisher Scientific, HPLC grade), acetonitrile (Fisher Scientific HPLC grade), ammonium chloride (Fisher Scientific, certified A.C.S. grade), hydroxocobalamin HCl (Sigma, 98%), pyridine (Aldrich, 99.8%, anhydrous), ethylene glycol (Aldrich, 99.8% anhydrous), silver nitrate (Fischer Scientific certified A.C.S. grade), reagent alcohol (Fischer Scientific (~90% EtOH, ~5% MeOH, ~5% isopropyl alcohol)) tosyl chloride (Aldrich, 98%). 8-bromoadenosine was purchased from Aldrich (98% claimed purity) and checked for purity, by decomposition point (210 ^{o}C) and ^{1}H NMR (Supplementary Material, Figure 2.S1), judged ~99% pure and, hence, was used as received. Zinc powder (Fisher Scientific, certified A.C.S. grade) was dried/activated in a 125 °C oven overnight prior to use. Distilled water was filtered through a Barnstead nano-pure filtration system. Ultrafiltration membranes (YC-05 nominal MW cutoff of 500) were purchased from Amicon (a division of Millipore Corporation) and preservatives (sodium azide and glycerin) were removed prior to use by soaking in distilled water for at least one hour with three changes of water during that time. The membranes were then placed in a 43 mm stirred filtration cell and used under 55 psig argon (General Air). Sephadex-SP C-25 (40-70 cm); (Sigma) columns were prepared as follows: 60 g of dry Sephadex was suspended in 600 ml of distilled water. The material was allowed to settle for a few minutes and the "fines" removed from the top of the

suspension by suction. The material was resuspended and this process repeated at least three times in order to remove most of the fines; the material was then allowed to soak for at least three hours. The material was washed with 2 M NaCl and then rinsed with distilled water at least three times. The material was poured into a 3.5 cm diameter column (resin height ~ 70 cm) and eluted with distilled water until the effluent contained no NaCl (determined by testing the eluant with $AgNO_3$). Amberlite XAD-2 (Supelco) columns were prepared as follows: 100 g of material was suspended in 500 mL MeOH and stirred until foaming ceased. The resin was then allowed to soak for ~20 min and the liquid was decanted. Next, 500 mL of distilled water was added to the resin, it was stirred for 10 min and then allowed to settle. This was repeated twice, and a final 500 mL of distilled water was added after which the resulting slurry was poured into a 3.5 cm diameter column (resin height ~ 25 cm).

4.2. Instrumentation and Equipment

UV-visible absorption spectra (± 1 nm) were recorded on a Hewlett-Packard Model 8452A UV-visible diode array spectrophotometer equipped with a thermoelectric Hewlett-Packard 89090A Peltier cell block temperature controller operating at 25.0 ± 0.1 °C. ^{1}H NMR spectra were recorded on an Inova-300 spectrometer operating at room temperature and were referenced internally to 0 ppm with TSP (D_2O) or referenced to the residual solvent peak at 2.50 ppm (DMSO-d_6). ^{13}C NMR spectra were recorded on an Inova-400 spectrometer operating at room temperature and were referenced internally to 0 ppm with TSP. HPLC was done with an HP 1050 HPLC with a 300 mm x 4.6 mm Alltech C-18 reverse phase column. Freeze drying was done with a Labconco Lyph-Lock 4.5 freeze drying system at –50 °C and at a vacuum of less than 250 μm Hg. Elemental

analysis was preformed by Atlantic Microlabs, with samples predried at 40 °C under vacuum. LSIMS (Liquid secondary ion mass spectrometry) was performed on a VG AutoSpec double-focusing mass spectrometer operating in the positive-ion detection mode. Samples were dissolved in *m*-nitrobenzyl alcohol matrices. Simulations were obtained using the Micromass program OpusV3.5X.

4.3. Handling of air sensitive and light sensitive materials

All air sensitive work was performed in a Vacuum Atmospheres inert atmosphere glovebox with an O_2 level < 2 ppm, as monitored by a Vacuum Atmospheres model AO 316-C oxygen analyzer.

Adenosylcobalamins are very photolabile; hence, all manipulations of cobalamins were done in a dark room exposed only to photographic quality red light, except when an oxygen-free atmosphere was necessary, in which case glassware was wrapped with aluminum foil and the needed work was done inside an inert atmosphere drybox.

4.4. Synthesis of 8-Bromoadenosine

For workers that might wish to synthesize their own 8-bromoadenosine, a detailed procedure is available (Supplementary Material, Section-S1).

4.5. Synthesis of 8-Methoxyadenosine

Synthesis of 8-methoxyadenosine was accomplished by a modified literature method [23] at room temperature. A detailed procedure is available (Supplementary Material, Section S-2).

4.6. Synthesis of 5'-tosyl derivatives

4.6.1 Synthesis of 5'-tosyladenosine

A detailed procedure of the synthesis and attempted isolation of 5'-tosyladenosine is available (Supplementary Material, Section S-4).

4.6.2. Synthesis of 5'-tosyl-8-methoxyadenosine

The next step in the synthesis involves replacing the 5'-OH on the adenosine with a tosyl leaving group. We used a method similar to an early literature tosylation [36], using only 1 equivalent of tosyl chloride to minimize tosylation at the 2'-OH and 3'-OH positions. A detailed procedure is available (Supplementary Material, Section S-4).

4.7 Synthesis of 5'-chloroadenosine

Details of the synthesis of 5'-chloroadenosine are available for the interested reader (Supplementary Material, Section S-5).

4.8. Preparation of 8-methoxyadenosylcobalamin and adenosylcobalamin

4.8.1. Preparation of 8-methoxyadenosylcobalamin,

The reduction of HOCbl was performed in a nitrogen atmosphere glovebox (O_2 < 2 ppm). First, 253 mg (1.67×10^{-4} mol) of $HOB_{12}HCl \cdot 7.5H_2O$ was dissolved in 60 mL of 10% NH_4Cl/water (deoxygenated by bubbling with a stream of nitrogen for 15 min) in a 100 mL round-bottomed flask containing a magnetic stir bar. To the flask, 2.16 g (3.42×10^{-2} mol, 200 equivalents) of activated Zn powder was added and stirring was carried out for ten minutes until the solution was greenish-black, indicating the formation of $Co(I)B_{12s}$. The flask was wrapped in aluminum foil to exclude light and capped with a rubber septum. The crude product from the synthesis of 5'-tosyl-8-methoxyadenosine (Section 4.6.1) was dissolved in 5 ml of methanol and added via syringe to the flask. Stirring was continued for 40 minutes at which point the flask was removed from the box and taken into a darkroom for workup. Observing the reaction solution at this time, being

careful to minimize light exposure, shows that the reaction solution was red. In the darkroom, the reaction mixture was suction filtered through a 30 mL medium glass frit to remove the unreacted zinc. The MeOH was then removed from the solution by rotary evaporation (~ 22 °C, < 10 mm Hg) for 30 minutes. The solution was applied to a 3.5 x 25 cm column of Amberlite XAD-2 and eluted with 500 mL of distilled water at ~ 5 mL/min. It is important to load the material slowly in order to ensure complete adsorption to the hydrophobic resin. The cobalamins bound to the column and the salts were washed away (demonstrated by testing for Cl^- in the eluant using a 0.2 M $AgNO_3$ solution). The cobalamins were then removed from the column by rinsing with 500 mL of 50/50 reagent alcohol / H_2O at a flow rate of ~ 3 mL/min. The alcohol was removed by rotary evaporation of the mixture (ca 30 minutes at ~ 22 °C, < 10 mm Hg).

The resulting solution was then filtered (under 55 psig argon) through an Amicon YC-05 filter (nominal MW cutoff 500). The cobalamins in the reaction mixture were substantially retained by this filter, as judged by the color and UV-visible spectrum of the filtrate, while low molecular weight components of the reaction mixture passed through (as demonstrated by HPLC of the mixture before and after filtration.) The retained cobalamins were suspended in 10 ml of distilled water and re-filtered through the same filter membrane. This rinsing process was repeated three to six times until HPLC showed no evidence of non-cobalamin impurities. The HPLC was monitored at 254 nm, where both nucleosides and cobalamins absorb, and also at 525 nm, where nucleosides do not absorb significantly but cobalamins do. Purification beyond the ultrafiltration proved necessary, as there were significant cobalamin impurities (~ 20%) shown by HPLC after filtration (Supplementary Material, Figure 2.S6).

These impurities were separated using a Sephadex-SP C-25 cation-exchange column. The cobalamin mixture was removed from the filtration cell with a minimum amount of water and applied to a 2.5 x 70 cm Sephadex-SP C-25 column and eluted with 1 L of distilled water at a rate of ~ 3 mL/min. One cobalamin side product eluted ahead of the desired compound[10] and some hydroxocobalamin remained at the top of the column. Fractions were analyzed by HPLC to ascertain their content and those containing only the desired product were combined in a foil wrapped flask and freeze dried (on a Labconco Lyph-Lock 4.5 freeze drying system at –50 °C and a vacuum of less that 250 μm Hg)[11] resulting in fluffy light-red material.

The yield was 102.1 mg (37%) with purity > 95% by HPLC (flow rate 2mL/min isocratic 85/15% H_2O/CH_3CN on a 300mm C-18 reverse phase column monitored at 525nm. RT~7.5 min), and ~ 90% by ^1H NMR (Figure 2.3) (D_2O referenced to TSP) δ 8.11(s, 1H) δ 7.17 (s, 1H) δ 6.95 (s, 1H) δ 6.25(d, 2H) δ 5.95 (s, 1H) δ 5.45(d, 1H). UV-visible spectrum (Supplementary Material, Figure 2.S1): λ max 318 (ε=1.40 x 10^4 M^{-1} cm^{-1}), 340 (ε=1.35 x 10^4 M^{-1} cm^{-1}), 376 (ε=1.18 x 10^4 M^{-1} cm^{-1}) and 524 (ε=8.80 x 10^3 M^{-1} cm^{-1}). LSIMS (Supplementary Material, Figure 2.S3) (m-nitrobenzyl alcohol matrix) calculated mw for $C_{73}H_{102}N_{18}O_{18}PCo{\cdot}H^+$=1609.7, found m/e ($M+H^+$)=1610.2. Other fragments were consistent with the literature [37] including 1329.5 ($M+H^+$–8-methoxyadenosyl group), 1069.5 ($M+H^+$–dimethylbenzimidazole-sugar), and 971.5

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[10] The impurity was an overtosylation product indicating that the tosylation step was not completely selective. Details are available (Supplementary Material, Section S-6).

($M+H^+$–dimethylbenzimidazole-sugar-phosphate-water). The ^1H NMR spectrum is very similar to that of AdoCbl, except that the resonance at δ 8.00 assigned in the literature to 8-H is not present (now replaced by the 8-MeO moiety) [31,32], and the resonance at δ 5.56, assigned to the proton at the A1' position, is shifted slightly to δ 5.45. The natural abundance ^{13}C NMR (Supplementary Material, Figure 2.S2) is also similar to that of AdoCbl [31] except, as expected, there is an additional peak at 60.6 ppm corresponding to the methoxy carbon, and the spectrum also shows the A8 peak shifted by ~10 ppm to 154.1 and A1' peak was shifted slightly upfield by ~ 0.8 ppm to 90.2 ppm.

Elemental analysis was performed by Atlantic Microlabs on a sample dried overnight at 40 °C under vacuum; $C_{73}H_{102}O_{18}N_{18}PCo\bullet 10H_2O$: calculated C, 48.99; H, 6.87; N, 14.09%. Found: C, 49.31; H 6.54; N, 13.52%. Elemental analyses on cobalamins are rare in the literature; the present analysis is quite satisfactory compared to those one can find in the literature.

4.8.2. The control reaction of the preparation of adenosylcobalamin using our synthetic method

As a test of our synthesis on another cobalamin, and especially its yield and the purity of the resultant product, AdoCbl was prepared analogously to 8-MeOAdoCbl. A detailed procedure is available (Supplementary Material, Section S-7).

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[11] One flask imploded during the freeze drying even though it showed no visible cracks. Hence, we recommended that all flasks used in the freeze drying process be inspected carefully under *polarized light* for cracks, strain, or other defects.

4.9. The determination of the rate of intramolecular cyclization of 5'-tosyladenosine and 5'-chloroadenosine

Rates of intramolecular cyclization are available (Supplementary Material, Section S-8 and Figure 2.S9.)

4.10. The thermolysis of 8-methoxyadenosylcobalamin

First, 5.0 mg (2.8×10^{-6} mol, corrected for the 10 waters of hydration) of 8-MeOAdoCbl was placed in a foil wrapped vial, taken into the drybox and dissolved in 0.7 mL of ethylene glycol (degassed by 3 freeze/pump/thaw/refill with argon cycles). Next, a portion of this solution (0.1 mL) was added to a foil-wrapped schlenk cuvette [35] and diluted to 3.0 mL with ethylene glycol, resulting in a ~ 1.2×10^{-4} M solution. The sealed cuvette was then removed from the drybox and taken to the UV-visible spectrophotometer, allowing exposure only to red light. UV-visible spectra were taken at regular time intervals over a period of 21 hours (Figure 2.3) and linear plots of ln $[(A_o - A_\infty)/(A - A_\infty)]$ vs. time (Supplementary Material, Figures 2.S4 and 2.S5), using the disappearance of the 376 nm peak and the appearance of the 474 nm peak, were made. The slope of these plots corresponds to a first-order rate constant for the reaction $k_{obs} = 8.5 \pm 0.2\ s^{-1}$.

5. Supplementary Material

Supplementary Material is available on the Elsevier website: http://www.elsevier.com/homepage/saa/jib. It is our plan to also publish the Supplementary Material in the Ph. D. thesis of K. M. Doll (Colorado State University, Fall 2002): UV-visible spectrum of 8-MeOAdoCbl (Figure 2.S1); ^{13}C NMR of 8-MeOAdoCbl in D_2O (Figure 2.S2); LSIMS of 8-MeOAdoCbl compared to a simulation (Figure 2.S3); Plot of ln $[(A_0-A_\infty)/(A-A_\infty)]$ vs time for the thermolysis of 8-MeOAdoCbl monitored at 376 nm (Figure 2.S4); Plot of ln $[(A_0-A_\infty)/(A-A_\infty)]$ vs time for the thermolysis of 8-MeOAdoCbl monitored at 474 nm (Figure 2.S5); ^1H NMR spectrum of 8-bromo-adenosine in DMSO-d_6 at room temperature (Figure 2.S6); ^1H NMR spectrum of 8-methoxy-adenosine in DMSO-d_6 at room temperature (Figure 2.S7); HPLC trace of a synthesis reaction solution after ultrafiltration (Figure 2.S8); Plot of ln (% starting material remaining) vs time for the cyclization of 5'-tosyladenosine (Figure 2.S9); The synthesis of 8-bromoadenosine (Section 2.S-1); The synthesis of 8-methoxyadenosine (Section 2.S-2); The synthesis and attempted crystallization of 5'-tosyladenosine (Section 2.S-3); The synthesis of 5'-tosyl-8-methoxyadenosine (Section 2.S-4); The synthesis of 5'-chloro adenosine (Section 2.S-5); Details on the cobalamin impurity that was removed in the SP-Sephedex column (Section 2.S-6); The synthesis of adenosylcobalamin by our synthetic method (Section 2.S-7); The determination of the rate of intramolecular cyclization of 5'-tosyladenosine and 5'-chloroadenosine (Section 2.S-8).

Acknowledgments

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Supplementary Material

The synthesis and characterization of 8-Methoxy-5'-deoxyadenosylcobalamin: a coenzyme B_{12} analog which, following Co-C bond homolysis, avoids cyclization of the 8-Methoxy-5'-deoxyadenosyl radical.

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Contribution from the Department of Chemistry, Colorado State University

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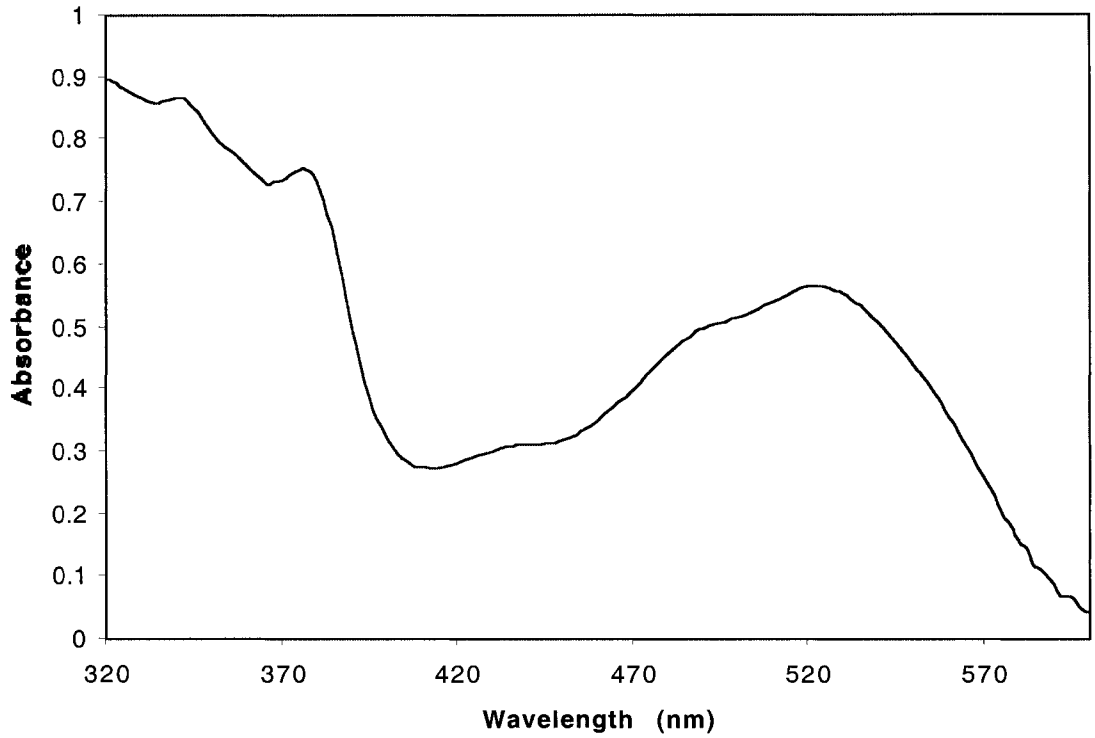


Figure 2.S1 The UV-visible spectrum of 8-MeOAdoCbl, λ max 318 (ε=1.40 x 10^4 M^{-1} cm^{-1}), 340 (ε=1.35 x 10^4 M^{-1} cm^{-1}), 376 (ε=1.18 x 10^4 M^{-1} cm^{-1}), and 524 (ε=8.80 x 10^3 M^{-1} cm^{-1}).

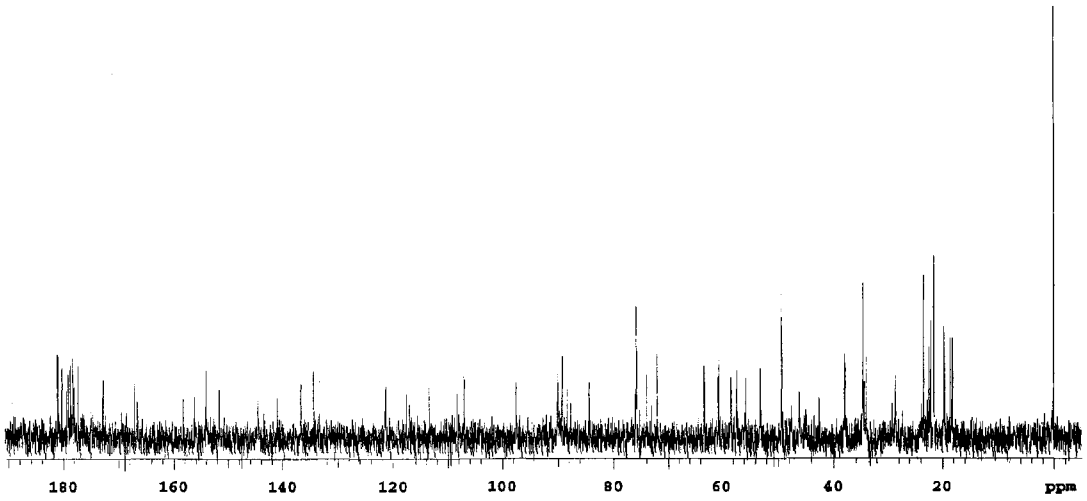


Figure 2.S2 A natural abundance ^{13}C NMR of 8.7 mg of 8-MeOAdoCbl in D_2O referenced to TSP. The above, 3/1 S/N spectrum proved more than sufficient to assign the ^{13}C NMR in close comparison to the assignments for AdoCbl [1].

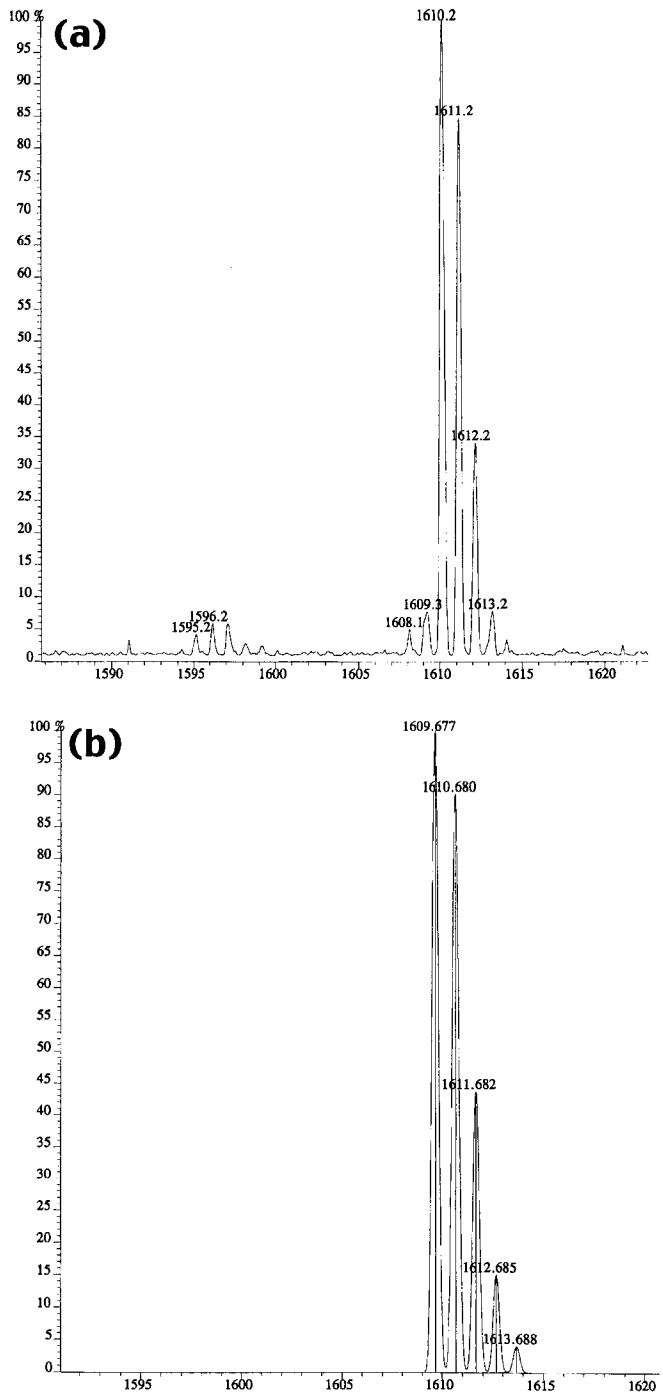


Figure 2.S3 (a) Enlarged L-SIMS MS (m-nitrobenzyl alcohol matrix); calculated mw for $C_{73}H_{102}N_{18}O_{18}PCo \cdot H^+$=1609.7, found m/e ($M+H^+$)=1610.2. (b) Computer simulated mass spectrum showing the excellent agreement between (a) and (b).

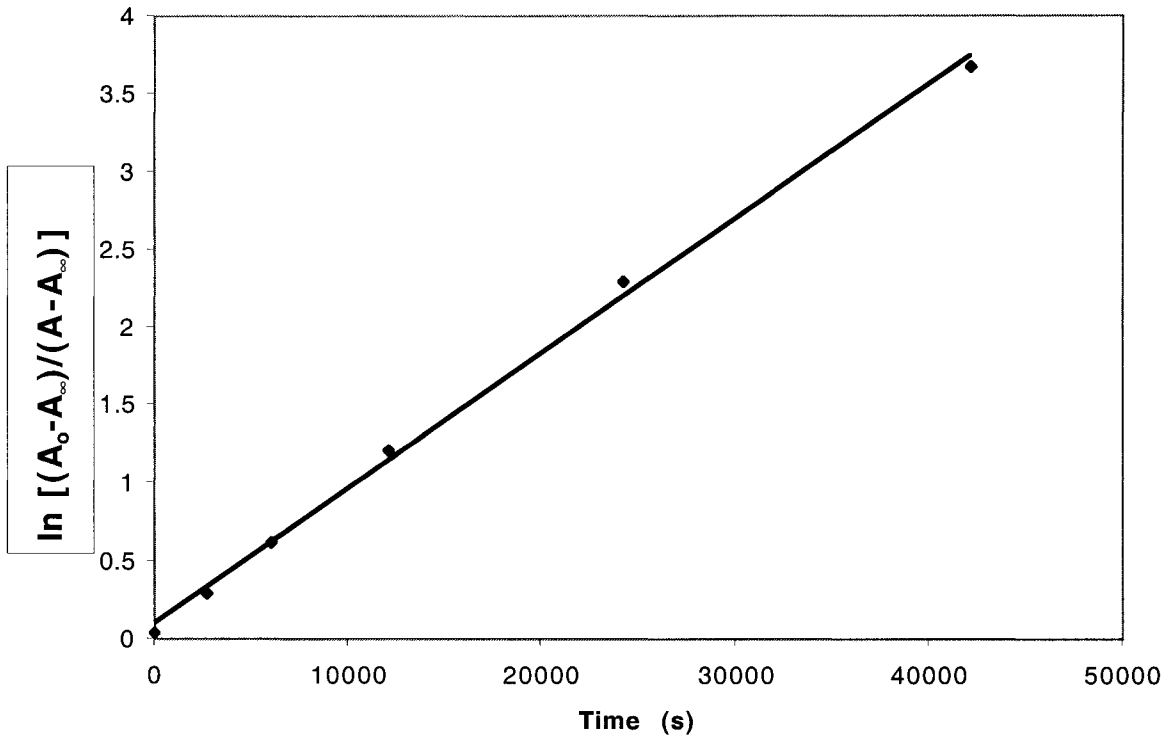


Figure 2.S4 Plot of ln $[(A_o-A_\infty)/(A-A_\infty)]$ vs. time for the thermolysis of 8-MeOAdoCbl monitored at 376 nm (section 4.10 in the main text). This plot's slope gives the first-order rate constant 8.6×10^{-5} s^{-1} and a $t_{1/2}$ of 2.2 hours

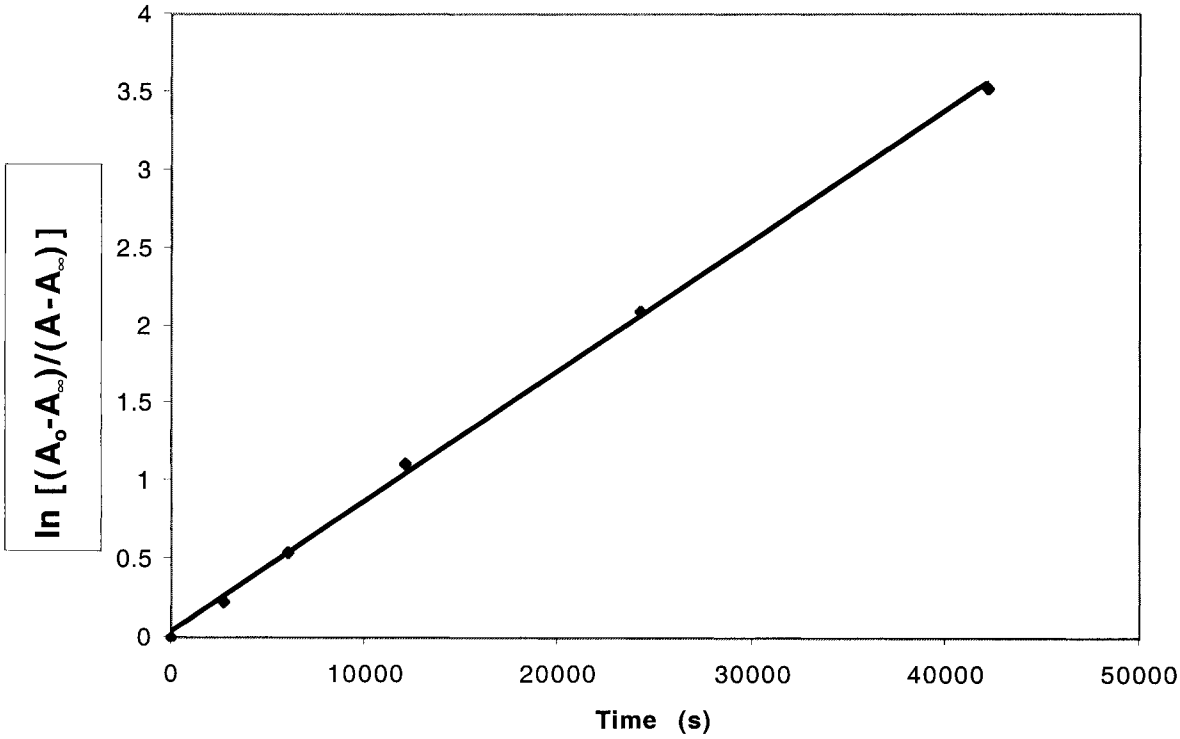


Figure 2.S5 Plot of ln $[(A_o-A_\infty)/(A-A_\infty)]$ vs. time for the thermolysis of 8-MeOAdoCbl monitored at 474 nm (section 4.9 in the main text). This plot's slope gives the first-order rate constant 8.3 x 10^{-5} $s^{-1}$and a $t_{1/2}$ of 2.3 hours.

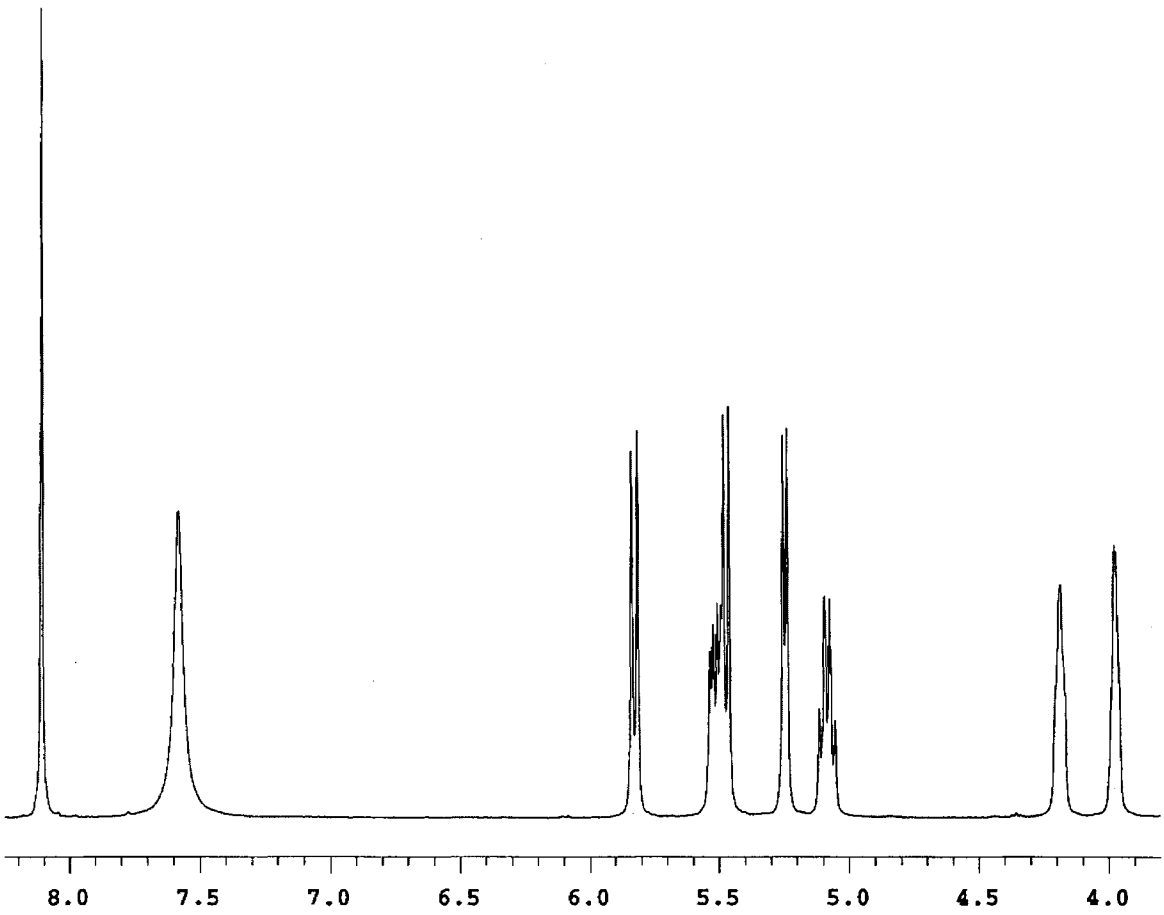


Figure 2.S6 The ^{1}H NMR (downfield region) of 8-bromoadenosine (DMSO-d6) δ 8.11(s, 1H, 2-H), δ 7.58 (s, 2 H, 6-NH_2), δ 5.82-5.84(d, 1H 1'-H), δ 5.46-5.54(m,2H 2'-and3'-OH), δ 5.24-5.25(m,1H, 5'-OH), δ5.08-5.09(q,1H. 2'-H), δ 4.18-4.19(br s, 1H, 3'-H), δ 3.97-3.98(m,1H,4'-H). The purity is ~99% as judged from the NMR spectrum (see section 4.5 in the main text).

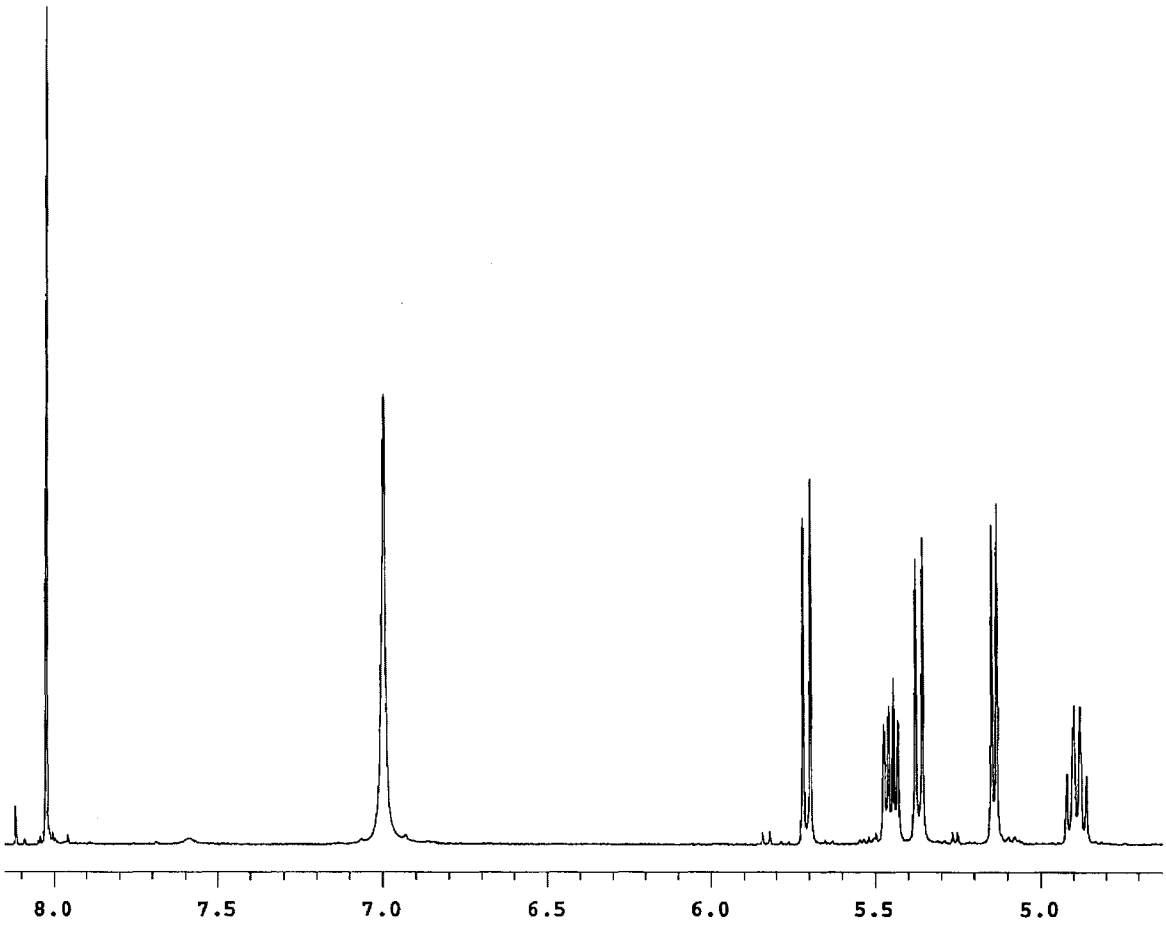


Figure 2.S7 The ^{1}H NMR (downfield region) of 8-methoxyadenosine (DMSO-d$_6$) δ 8.02(s, 1H 2-H), δ 6.99 (s, 2H 6-NH$_2$), δ 5.70-5.72(d, 1H, 1'H) δ 5.5-5.7 (t,1H, 5'- OH) δ 5.36-5.37(d, 1H, 2'-OH), δ 5.13-5.15 (d,1H,3'-OH) δ 4.88-4.90 (q, 1H, 2'-H). The purity is ≥96% as judged from the NMR spectrum (see section 4.5 in the main text).

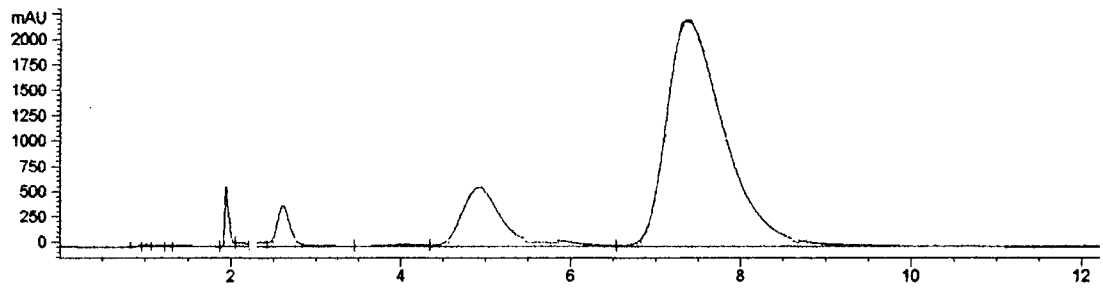


Figure 2.S8 HPLC trace (detector at 254 nm) of a synthesis reaction solution after ultrafiltration, but before the SP-Sephadex column chromatography. The 8-MeOAdoCbl peak (retention time ~7.5 min) area is about 80% of the total area. The main impurity, (retention time ~4.9 min and ~16% of the total area) is an overtosylated 8-MeOAdoCbl based on LSIMS. The small impurity peak (retention time ~2.5 min and ~3% of the total area) is a small amount of remaining nucleoside.

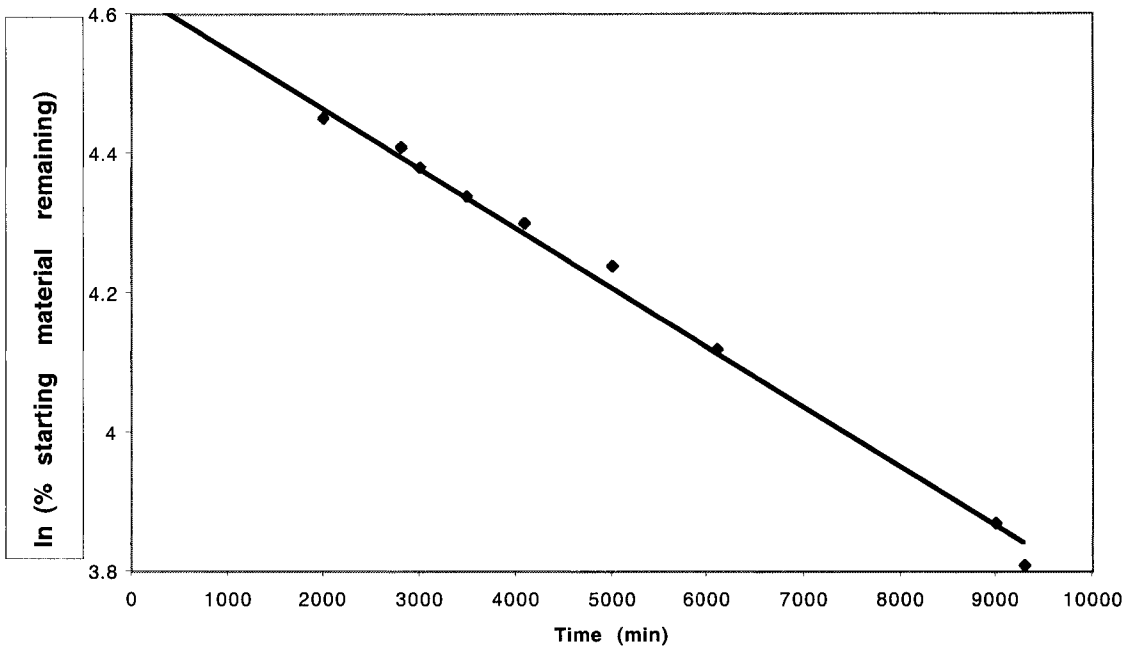
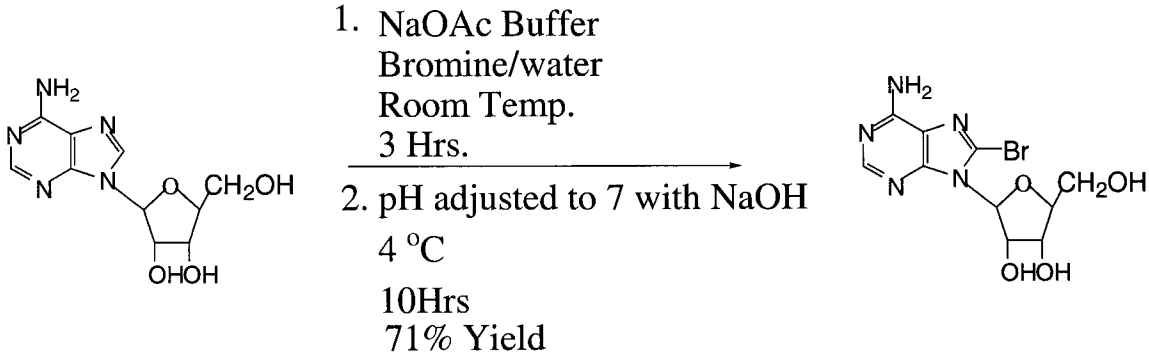


Figure 2.S9 Plot of ln (% starting material remaining) vs. time for the cyclization of 5'-tosyladenosine (Supplementary Material, Section S-4). This plot's slope gives the first-order rate constant 8.3 x 10^{-5} $min^{-1}$and a $t_{1/2}$ of 5.8 days.

Section 2.S-1

The synthesis of 8-bromoadenosine.



Before it was available commercially, we synthesized 8-bromoadenosine according to the literature procedure [2]; yield 71%, (lit yield 74.5%); purity >98% by HPLC (Alltech Versapack C-18 column, isocratic at 7% CH_3CN 93% H_2O, 1ml/min, retention time 22.4 min.) m.p. 226-228 °C (preheated block) (lit m.p. >200°C dec.). NMR; (DMSO-d_6) δ 8.11(s, 1H, 2-H), δ 7.59 (s, 2 H, 6-NH_2), δ 5.79-5.94(d, 1H 1'-H), δ 5.41-5.64(m, 2H 2'-and3'-OH), δ 5.20-5.35(m, 1H, 5'-OH), δ 5.04-5.16(q, 1H. 2'-H), δ 4.15-4.27(br s, 1H, 3'-H), δ 3.94-4.07(m, 1H, 4'-H), δ 3.47-3.78(m, 2H 5'-H). The observed ^{1}H NMR spectrum is consistent with the literature spectrum [3]. Elemental analysis calculated for $C_{10}H_{12}BrN_5O_4$; C, 34.70 H, 3.49; Br, 23.08; N, 20.23; O, 18.49%. Found: C, 34.66; H 3.48; Br, 23.29; N, 20.49; O, 18.33%. It is conceivable that bromination could occur at the 2 position rather than the 8 position; however, crystal structures of 8-bromoadenosine [4] and its isopropylidene protected derivative [5] (prepared using the standard method for bromination in the 8 position [3]) have been published and show unequivocally that bromination occurs, as desired, at the 8 position.

Section 2.S-2

The synthesis of 8-methoxyadenosine.

The required 8-Methoxyadenosine was synthesized by stirring 8-bromoadenosine (5g, 14.5 mmol) with a 10-fold excess of sodium methoxide in 800 mL of methanol at 25 °C. The starting material would not completely dissolve in this amount of MeOH, but the 8-bromoadenosine gradually dissolved as conversion to product proceeded. Product formation was monitored by removing ~0.1 mL aliquots of reaction solution and testing by HPLC (flow rate 1 mL/min isocratic 90/10% H_2O/CH_3CN on a 300 mm C-18 reverse phase column monitored at 254 nm). The retention time of the 8-MeOAdo product was ~10.5 min, while the retention time of the starting material was ~15.2 min. The reaction was completed in ~11 hours. It is important that all of the starting material be allowed to react, as separation of product and starting material proved difficult. The relatively harsh conditions used in the literature preparation of 8-MeOAdo [6], involving refluxing in $MeOH/MeO^-$ for 18 hours, proved unnecessary. (It is known [7] that basic conditions can cause hydrolysis of the N-glycosidic linkages of nucleosides and some degradation of our product was observed even at room temperature. This may be even more of a problem in the 8-methoxy compound than in adenosine, because of the electron donating resonance effect of the methoxy group. Hence it appears important to avoid unnecessarily vigorous conditions.) The reaction mixture was neutralized with concentrated HCl and the solvent removed by rotary evaporation (40 °C, <10 mm Hg). Purification was achieved using a 3.5 x 25 cm column of Amberlite XAD-2. The mixture of solids from the reaction was ground to a fine powder in a mortar and applied to the top of the column, which was then eluted with water. Fractions were collected and analyzed for NaCl (using

$AgNO_3$) and other impurities (by HPLC). NaCl came off first, along with unidentified material. The product came off very slowly, requiring about 4 L of eluent for complete removal. A brown band remained on the column and could be removed by rinsing the column with 50/50 CH_3CN/H_2O. It contained 8-methoxyadenine identified by ^1H NMR. The yield was 1.544 g (36%) with purity >96% by HPLC (flow rate 1 mL/min, isocratic 90/10% H_2O/CH_3CN, 300 mm C-18 reverse phase column monitored at 254 nm, retention time ~10.5 min.) and ^1H NMR (Supplementary Material, Figure S7) (DMSO-d_6) δ 8.02(s, 1H 2-H), δ 6.99 (s, 2H 6-NH_2), δ 5.70-5.72(d, 1H, 1'H) δ 5.5-5.7 (t, 1H, 5'-OH) δ 5.36-5.37(d, 1H, 2'-OH), δ 5.13-5.15 (d, 1H, 3'-OH) δ 4.88-4.90 (q, 1H, 2'-H), δ 4.1(br s, 4H, OMe and 3'H), δ 3.9 (br s, 1H, 4'-H), δ 3.4-3.7 (m, 2H, 5'H_2).

Section 2.S-3

The synthesis and attempted isolation of 5'-tosyladenosine.

5'-Tosyladenosine was made for use in a control experiment synthesizing AdoCbl (instead of 8-MeOAdoCbl). It was prepared in an analogous manner to 5'-tosyl-8-methoxyadenosine (section 4.6.1). In an attempted isolation of the tosylated adenosine, the thick, dark yellow crude product solution (with the pyridine removed by rotary evaporation as in section 4.6.1) was added dropwise to 25 mL of cold (0 °C) acetone and allowed to crystallize in the freezer (-4 °C) overnight. White and yellow crystals were harvested (135 mg) by filtration through a Buchner funnel and recrystallized by dissolving in 0.8 mL warm water, adding 9.2 mL acetone, and allowing to crystallize in the freezer for 48 hours. White crystals were obtained and a ^{1}H NMR was taken (d_6-DMSO referenced to residual solvent) δ 8.71(s, 1H) δ 8.49(s, 1H) δ 5.95 (d, 1H) δ 4.52 (t, 2H) δ 4.17 (t, 1H) δ 3.99(q, 1H) δ 3.63(m, 2H). This spectrum is similar but not identical to the published ^{1}H NMR of N^3,5'-*cyclo*adenosine [8].

Section 2.S-4

The synthesis of 5'-tosyl-8-methoxyadenosine.

In a 50 mL round-bottomed flask with a magnetic stir bar, 0.248 g of 8-methoxyadenosine (0.833 mmol, 5 equivalents relative to the amount of $HOB_{12}{\cdot}HCl{\cdot}7.5H_2O$ used in Section 4.8.1) was dissolved in 30 mL of pyridine. The solution was cooled to -10 °C in an ice/salt bath while being purged with nitrogen using two needles inserted through a rubber septum. Next, 0.158 g of tosyl chloride [0.833 mmol, 1 equivalent (to minimize over tosylation) compared to the amount of 8-MeOAdo used)] was added, the septum was replaced and nitrogen purging continued for another ten minutes. The ice/salt bath was replaced with an ice bath and stirring was carried out for four hours. Over this time, the solution turned from clear to dark yellow. The ice bath was then removed and stirring continued for an additional eight hours. Water (5 ml) was added in order to destroy any unreacted tosyl chloride. (This step, done before the pyridine is removed, is crucial; without it, multiple minor cobalamin impurities are observed by HPLC after the next step of the synthesis.) The pyridine was removed by rotary evaporation (<10 mm Hg, room temperature) and the resultant thick, dark yellow, and syrupy crude product was used in the cobalamin alkylation step (Section 4.8.1).

Section 2.S-5

The synthesis of 5'-chloroadenosine.

5'-Chloroadenosine, used for the determination of the intermolecular cyclization rate, (section 4.9) was prepared by the literature method of Kikugawa and Ichino [9]. Yield: 60%, (lit. yield 75%); Purity > 98% by HPLC (Hamilton PRP-1 column, isocratic at 15%CH_3CN/85%H_2O, 2 ml/min, rt. 6.7 min; NMR (d_6-DMSO) δ 8.34 (s, 1H), δ 8.15 (s, 1H), δ 7.32 (s, 2H), δ 5.92 (d, 1H), δ 5.59 (d, 1H), δ 5.45 (d, 1H), δ 4.74 (q, 1H), δ 4.21 (q, 1H), δ 4.07 (q, 1H), δ 4.04-3.80 (m, 2H). NMR showed that the product was contaminated with approximately 4% HMPT, according to its characteristic resonance at δ 3.35 ppm.

Section 2.S-6

Details on the cobalamin impurity that was removed in the SP-Sephadex column.

The main impurity that eluted ahead of the desired product (HPLC retention time (4.9 min with a flow rate 2 mL/min isocratic 85/15% H_2O/CH_3CN on a 300 mm C-18 reverse phase column monitored at 525 nm) was isolated by freeze drying and subjected to LSIMS. It gave a molecular ion peak at m/e 1747.5 which corresponds to an 8-methoxy-5'-deoxyadenosylcobalamin in which either the 2' or the 3' hydroxyl group has been tosylated (calculated 8-methoxy-5'-deoxy-X'-OTs-adenosylcobalamin $+H^+$ m/e = 1747.7), indicating that, as expected, the tosylation of 8-MeOAdoOH was not completely selective. A ^1H NMR of the impurity was taken showing a similar aromatic region (D_2O referenced to TSP) δ 8.06(s, 1H) δ 7.03 (s, 1H) δ 6.33 (s, 1H) δ 6.29(d, 2H) δ 5.97 (s, 1H) δ 5.41(d, 1H). As expected, no similar problem was noted in the adenosylcobalamin synthesis (Section 4.8.2) due to conformationally increased tosylation selectivity [10].

Section 2.S-7

The synthesis of adenosylcobalamin by our synthetic method.

Adenosine was tosylated with tosyl chloride in pyridine, the pyridine was removed, and the AdoOTs was added in 5-fold excess to a Co(I)Cbl solution generated by the reduction of HOCo(III)Cbl with zinc, all following the same procedures used in the 8-MeOAdo synthesis. The product was 96% pure by HPLC after using the Amberlite XAD-2 column and the Amicon ultrafilter. The product was removed from the ultrafiltration membrane by dissolving it in 10 ml of water, freeze drying, and finally drying at 0.01 mm Hg and 40 °C overnight. The yield was 93 mg (70%) with ~96% purity by HPLC (Millipore Waters Model 510 HPLC, Hamilton PRP-1 column, isocratic at 15% CH_3CN/85% H_2O, 2 mL/min retention time ~6.7 min). UV-visible spectrum (in deionized water): λ-max 524 nm (ε= 1.10 x 10^4 M^{-1} cm^{-1}), 376 nm (ε= 1.48 x 10^4 M^{-1} cm^{-1}), and 338.5 nm (ε= 1.74 x 10^4 M^{-1} cm^{-1}). ^1H NMR (D_2O): δ 7.97(s, 1H) δ 7.77(s, 1H) δ 6.93(s, 1H) δ 6.70 (s, 1H) δ 6.00 (d, 2H) δ 5.70(s, 1H) δ 5.33 (d, 1H). The ^1H NMR spectrum of the synthetic sample is identical with a spectrum recorded of authentic material purchased from Sigma. FAB-MS (fast atom bombardment mass spectroscopy) (m-nitrobenzyl alcohol matrix) calculated mw for $C_{72}H_{100}O_{17}N_{18}PCo \cdot H^+$=1579.59, found m/e ($M+H^+$)= 1579.59, ($M+H^+$-5'-deoxyadenosyl)=1330. Other fragment peaks were consistent with the literature [11,12].

Section 2.S-8

The determination of the rate of intramolecular cyclization of 5'-tosyladenosine and 5'-chloroadenosine.

Rates of cyclization at room temperature (~22 °C) were determined by monitoring 0.001 M solutions of the nucleosides in 1/1 MeOH/H_2O using HPLC (Hamilton PRP-1 reversed phase column, isocratic at 30/70 CH_3CN/H_2O, 2 mL/min monitored at 254 nm, retention time of the starting material 9.1 min, retention time of the cyclized material 13.4 min). Rate constants were determined by plotting ln (% starting material remaining) vs. time, k being defined as minus the slope of the resulting linear plot (Supplementary Material, Figure S9, vida supra). The half-life for cyclization of 5'-tosyladenosine is $t_{1/2}$ = 5.8 ± 0.2 days at ~ 22 °C. After two weeks, there was no sign of decomposition or cyclization of 5'-chloroadenosine, $t_{1/2} \geq 190$ days assuming a ≥ 5% detection limit.

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CHAPTER 3

The First *Experimental* Test of the Hypothesis that Enzymes Have Evolved to Enhance Quantum Mechanical Tunneling

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This chapter is intended for submission to *Journal of the American Chemical Society* with only modifications to the margins and figure numbering for compliance with that journal's requirements. This chapter reports the first *experimental* test of Klinman's hypothesis: that enzymes may have evolved to enhance quantum-mechanical (QM) tunneling.[1]

QM tunneling has been established in the $H^{\bullet}$ abstraction reactions mediated by the AdoCbl-dependent enzyme Methylmalonyl-CoA Mutase[2] (MMCoA); there is also evidence for tunneling in other AdoCbl-dependent enzyme reactions. To establish whether or not the enzyme is enhancing or otherwise changing the degree of tunneling, a comparison must be made to the same reaction in the enzyme-free system. This chapter reports a study of the $H^{\bullet}$-atom abstraction from ethylene glycol substrate in solution by Ado• produced from the thermolysis of AdoCbl. The same well-established QM tunneling criteria used in the enzyme system (kinetic isotope effect > 4.6 at 80°C, activation energy difference > 1.2 kcal mol^{-1}, pre-exponential factor ratio < 0.7)[3] have been measured for the AdoCbl solution system, allowing a comparison to the enzymic data.

In addition, the AdoCbl analog, 8-methoxy-5'-deoxy-adenosylcobalamin (8-MeOAdoCbl), whose synthesis is presented in chapter two of this dissertation, has also been studied. This molecule eliminates the 8 to 5' cyclization reaction of the $Ado^{\bullet}$ thereby simplify the system and the data analysis, allowing a more direct measurement of the kinetic isotope effect by HPLC-MS. The results for the three tunneling criteria for 8-MeOAdoCbl proved to be the same within experimental error as those for AdoCbl.

The resultant data allows the first experimental test of Klinman's hypothesis. Comparing the enzyme-free vs enzyme data leads to the conclusion that AdoCbl-dependent enzyme*s do not enhance QM tunneling*. Instead, they simply exploit the same degree of QM tunneling already present in the Ado$^{\bullet}$ hydrogen-abstraction reaction. There is no discernable change in the amount of tunneling with, or without, the enzyme.

Abstract

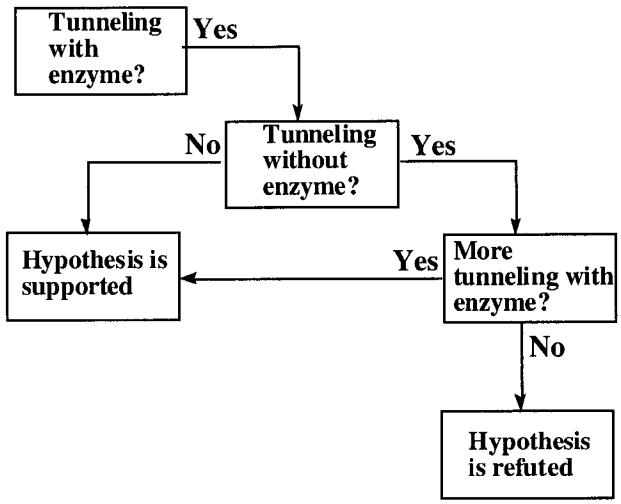
Klinman's hypothesis—that "the optimization of enzyme catalysis may entail the evolutionary implementation of chemical strategies that increase the probability of quantum-mechanical tunneling"—is *experimentally* tested herein for the first time. The system employed is the key to being able to provide this first experimental test of the "enhanced tunneling" hypothesis, one that requires a comparison of the three criteria diagnostic of tunneling (vide infra) for the same, or nearly the same, reaction with *and without* the enzyme. Specifically, studied herein are the adenosylcobalamin (AdoCbl; also known as Coenzyme B_{12})-dependent diol dehydratase model reactions of (i) H(D)$^{\bullet}$-atom abstraction from ethylene glycol-d_0 and ethylene glycol-d_4 solvent by 5'-deoxyadenosyl radical (Ado$^{\bullet}$), and (ii) the same H$^{\bullet}$ abstraction reactions by the 8-methoxy-5'-deoxysdenosyl radical (8-MeOAdo$^{\bullet}$). The Ado$^{\bullet}$ and 8-MeOAdo$^{\bullet}$ radicals are generated by Co–C thermolysis of their respective precursors, AdoCbl and 8-MeOAdoCbl. Deuterium kinetic isotope effects (KIEs) of the H$^{\bullet}$(D$^{\bullet}$)-abstraction reactions from ethylene glycol have been measured over temperature range of 80-120 $^{\circ}$C: KIE = 12.4 ± 1.1 at 80 $^{\circ}$C for Ado$^{\bullet}$ and KIE = 12.5 ± 0.9 at 80 $^{\circ}$C for 8-MeOAdo$^{\bullet}$ (values more than twice that of the predicted maximum primary ground-state zero-point energy (GS-ZPE) KIE of 4.6 at 80 $^{\circ}$C). From the temperature dependence of the KIEs, zero-point activation energy differences ($[E_D-E_H]$) of 3.0 ± 0.3 kcal mol^{-1} for Ado$^{\bullet}$, and 2.1 ± 0.6 kcal mol^{-1} for 8-MeOAdo$^{\bullet}$, have been obtained, both of which are significantly larger than the GS-ZPE maximum of 1.2 kcal mol^{-1}. Pre-exponential factor ratios (A_H/A_D) of 0.16 ± 0.07 for Ado$^{\bullet}$ and 0.5 ± 0.4 for 8-MeOAdo$^{\bullet}$ are observed, both of which are significantly less than the 0.7 minimum for non-tunneling behavior. Overall, the data

provide strong evidence for quantum mechanical tunneling in the Ado$^{\bullet}$ and 8-MeOAdo$^{\bullet}$ mediated H$^{\bullet}$ abstraction reactions from ethylene glycol. More importantly, a comparison of these enzyme-free tunneling data to the same KIE, (E_D-E_H) and A_H/A_D data for a closely related, Ado$^{\bullet}$ mediated, H$^{\bullet}$ abstraction reaction from a primary CH_3- group in AdoCbl-dependent methylmalonyl-CoA mutase shows the enzymic and enzyme-free data sets *are identical within experimental error*. The irrefutable conclusion is that at least this adenosylcobalamin-dependent enzyme has not evolved to enhance quantum mechanical tunneling. Instead, this enzyme simply exploits the identical level of quantum-mechanical tunneling that is available in the enzyme-free, solution-based H$^{\bullet}$ abstraction reaction. The results also require a similar if not identical barrier shape (i.e., barrier width and height) for the H$^{\bullet}$ abstraction both within, and outside of, the enzyme.

Introduction

An intriguing[4] but controversial[5-7] hypothesis dating back to 1989[8,9] if not before[10] is Klinman's suggestion that "the optimization of enzyme catalysis may entail the evolutionary implementation of chemical strategies that increase the probability of tunneling and thereby accelerate the reaction rate."[4] Restated, enzymes may have evolved to enhance quantum mechanical (QM) tunneling.[10,11,12] This "enhanced tunneling" hypothesis merits careful scrutiny since the availability of many low-frequency motions in proteins[13-20] can change interatomic distances by distances by 0.2 to ca. 1.5 Å or more,[14] *conceivably* resulting in enhanced tunneling between thereby closer reaction sites (e.g., H• atom-transfer sites, for example, vide infra)[21-28,29] Science proceeds through the *disproof* of alternative hypotheses[30] ("for exploring the unknown, there is no faster method").[30] Two alternative hypotheses are: that enzymes do not operate to change substantially the shape of barriers (e.g., the width vs just the height) and, hence, do not enhance tunneling, or that tunneling is actually decreased in the enzyme as a result of a (seemingly unlikely) increase in the reaction barrier *width* as evolution has led to a decrease in the barrier *height.*[31,32]

On reflection, we and others[5,33,34] realized that a very simple yet definitive test of the enhanced-tunneling hypothesis is possible at least in principle by a comparison of degree of tunneling for the same reaction with, and without (i.e., outside of) the enzyme, Scheme 3.1.



Scheme 3.1 A simple three-step procedure testing the hypothesis that enzymes have evolved to enhance quantum-mechanical tunneling. It is important that the same substrate, or at least a substrate as similar as possible, be used when following this procedure in order to avoid results that are complicated by substrate-dependent tunneling.

However, an *experimental* test of the enhanced tunneling hypothesis has not appeared until now due to the difficulty of finding a system where the identical, or at least a very similar, reaction such as a H• transfer reaction, can be studied experimentally both with *and without* the enzyme.

Upon reflection we realized that the Coenzyme B_{12} (5'-deoxyadenosylcobalamin; AdoCbl) -dependent diol dehydratase reaction is a nearly ideal system in which to test the enhanced tunneling hypothesis. In this enzymic reaction, enzyme-accelerated Co–C homolysis of AdoCbl[35] generates an at least formal Ado•,[36] and this Ado• then abstracts a H• from ethylene glycol substrate (d_0-$HOCH_2CH_2OH$ or its deuterated-d_4 analog,

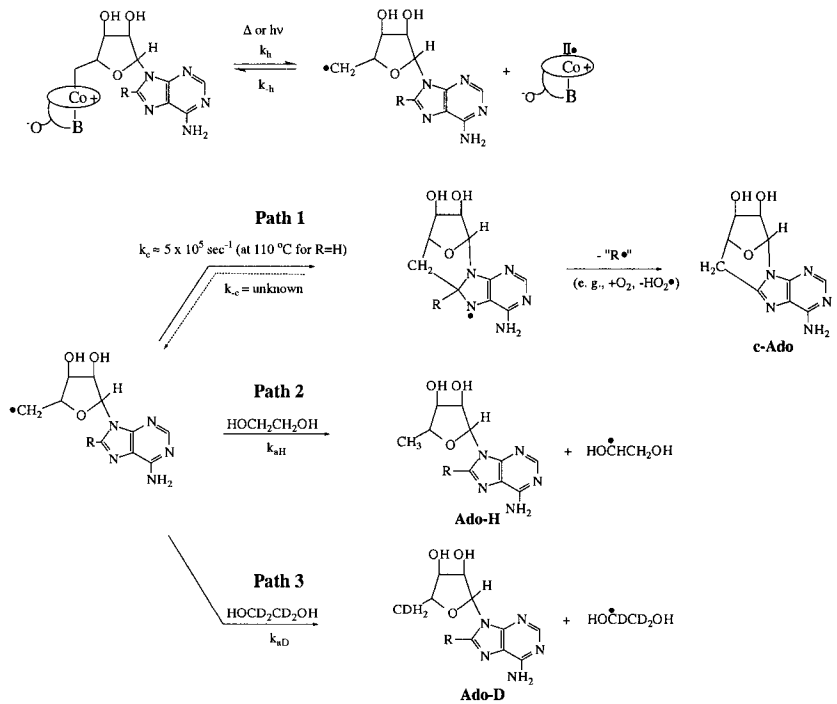
$HOCD_2CD_2OH$). Significantly, this identical albeit much slower reaction is known in solution when enzyme-*free* AdoCbl is thermolyzed in ethylene glycol solution.[37] Although the needed conclusive data probing tunneling for the enzyme diol dehydratase itself do not yet exist, a *very similar* H• abstraction from a primary C-H bond has been studied recently by Banerjee and co-workers for AdoCbl-dependent methylmalonyl-CoA mutase.[2] The tunneling literature[31] indicates that little difference is expected in the degree of tunneling for these two systems given their similar C-H bond dissociation energies (BDEs), $HOCH_2CH_2OH$, BDE (C-H) = 91.1-95 kcal mol^{-1} [38,39] vs methylmalonyl-CoA H_3C-R estimated BDE (C-H) ~ 92-93 kcal mol^{-1} [39,40]. The methylmalonyl-CoA mutase system is furthermore ideal in that the three needed criteria for QM tunneling first developed by Kwart,[41] and then refined by Kreevoy,[3] are available for methylmalonyl-CoA mutase,[2] namely: (i) a deuterium kinetic isotope effect (KIE; k_H/k_D) significantly larger than the ground-state zero-point-energy difference (GS-ZPE) maximum KIE(6.4 at 20 °C, or 8.9 at 20 °C when one includes secondary isotope effects); (ii) an activation energy difference (E_D-E_H) greater than 1.2 kcal mol^{-1}, and (iii) a ratio of pre-exponential factors (A_H/A_D) less than 0.7. The first criterion simply requires the measurement of the KIE while the other two criteria can be obtained from the measurement of the isotope effect as a function of temperature and, then, an Arrhenius plot of ln KIE vs 1 / temperature.[42] The value E_D-E_H is obtained from the slope while A_H/A_D is obtained from the intercept. The data from Banerjee's labs for methylmalonyl-CoA mutase are: a KIE of 35.6 at 30 °C, an activation energy difference (E_D-E_H) of 3.41 ± 0.07 kcal mol^{-1} and a pre-exponential factor ratio (A_H/A_D) of 0.078 ± 0.009. Each of these values is well outside the aforementioned GS-ZPE limits, indicative of substantial

tunneling in the H• abstraction reaction from the H_3C-R group of methylmalonyl-CoA by an incipient Ado•.[43,44] This same system was also studied by theoretical methods which require tunneling to fit the data.[45,46]

Herein we report the first experimental test of the "enzyme-enhanced tunneling" hypothesis. We have obtained the three Kreevoy criteria for AdoCbl and 8-MeO-AdoCbl Co–C bond thermolysis, to produce Ado• and 8-MeO-Ado•, respectively, followed by their H• abstraction from mixture of $HOCH_2CH_2OH$-d_0 and $HOCD_2CD_2OH$-d_4 over a temperature range of 80 °C to 120 °C. Comparing the results of these enzyme-free tunneling data to the same KIE, (E_D-E_H) and A_H/A_D data for AdoCbl-dependent methylmalonyl-CoA mutase (vide supra) reveals that the enzyme-free and enzymic data sets *are identical within experimental error*. The irrefutable conclusion is that at least this adenosylcobalamin dependent enzyme has *not* evolved to enhance quantum mechanical tunneling in its H• abstraction reactions. Instead, this enzyme simply exploits the identical level of quantum-mechanical tunneling that is already present in the enzyme-free, solution-based H• abstraction reaction. The results also support a similar if not identical barrier shape (i.e., width and height) for the H• abstraction both inside, and outside of, the enzyme.[32]

Results and Discussion

The systems studied are shown in Scheme 3.2: the AdoCbl system, where R=H, and the 8-MeOAdoCbl system, where R=MeO.



Scheme 3.2 The reaction scheme for the Adenosylcobalamin (AdoCbl; R = H) and, separately, 8-methoxy-5'-deoxy-adenosylcobalamin (8-MeOAdoCbl; R = MeO) homolysis in ethylene glycol solution.

The AdoCbl thermolysis reaction in ethylene glycol (Scheme 3.2, R=H) has been well studied.[47] The homolysis reaction of the Co–C bond is known to produce Co(II)Cbl• and Ado•, and the associated rate law has been derived using the steady state approximation for Ado•.[48] The Ado• radical is known to react via three primary pathways:[49] (i) it cyclizes (path 1 in Scheme 3.2), and ultimately leads to the stable product[50] 8,5'-anhydroadenosine (c-Ado);[37,47,51,52] (ii) it abstracts a H-atom from ethylene glycol solvent (path 2 in Scheme 3.2) yielding the other observed product, 5'-deoxyadenosine (Ado-H);[13,37] or (iii) it returns to Co(II)Cbl• to reform AdoCbl. Because the intramolecular cyclization reaction of Ado• is isotope *insensitive*, the amount of c-Ado serves as a convenient, built-in internal standard for the ratio of the isotope *sensitive* H vs D abstraction products from solvent, Ado-H and Ado-D, Scheme 3.2. The desired KIE is readily obtained via equation 3.1 where r(H) is the product ratio, c-Ado/Ado-H, observed in non-deuterated ethylene glycol solvent and r(D) is the corresponding product ratio observed in deuterated ethylene glycol (a derivation is provided in Section 3.S-6, derivations 1 and 2, of the Supporting Information).

$$KIE = \frac{k_{aH}}{k_{aD}} = \frac{(c\text{-}Ado/AdoD)_{d_4\,solution}}{(c\text{-}Ado/AdoH)_{d_0\,solution}} = \frac{r(D)}{r(H)}$$

Equation 3.1

Experimentally and since AdoCbl homolysis is slow at room temperature ($t_{1/2}$ of 22 years at 25 °C), solutions of AdoCbl in ethylene glycol were thermolyzed in 10 °C increments from 80-120 °C, temperatures where AdoCbl homolysis proceeds at a convenient rate. The thermolysis reaction proceeded as shown in Scheme 3.2 exhibiting

clean conversion to Co(II)Cbl$^{\bullet}$ by UV-visible spectroscopy (Figure 3.S1, Supporting Information) plus the expected products by HPLC (Figure 3.S2-3.S3, Supporting Information).[47] When AdoCbl was thermolyzed in ethylene glycol-d_4 the product ratios changed as expected: less Ado-D, and correspondingly more c-Ado, were observed consistent with a substantial KIE for Ado$^{\bullet}$ abstraction of a H- or D–atom from the C–H(D) bond[53,54,55] of ethylene glycol: a KIE ranging from = 8.1 ± 0.7 *at 120 °C* to 12.4 ± 1.1 *at 80 °C*, Figure 3.3. These isotope effects are more than twice the predicted, maximum primary GS-ZPE KIEs of 3.9 at 120 °C and 4.6 at 80 °C calculated using a version of the Bigeleisen equation: $k_H/k_D \approx \exp[hc/2k_BT\ (\nu_{CH}-\nu_{CD})]$ (and inserting a C–H stretching frequency of 2891 cm^{-1} for ethylene glycol and a C–D stretching frequency of 2137 cm^{-1} for ethylene glycol-d_4).[56,57] The activation energy difference, $[E_D-E_H]$ = 3.0 ± 0.3 kcal mol^{-1}, was calculated from the slope of a ln KIE vs 1/T Arrhenius plot, Figure 3.1, and the ratio of pre-exponential factors, A_H/A_D = 0.16 ± 0.07, was calculated from the intercept of that plot. Both of these criteria also indicate substantial tunneling in the enzyme-free, Ado$^{\bullet}$-mediated H$^{\bullet}$ abstraction reaction.

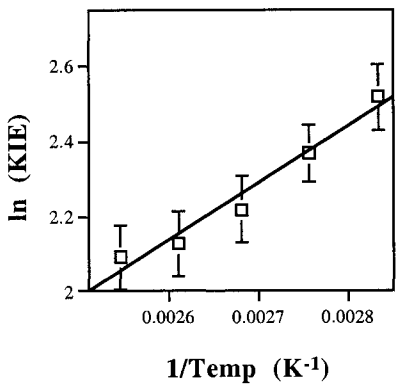


Figure 3.1 A plot of ln KIE vs 1/T for the AdoCbl thermolysis. From the slope and intercept, the activation energy difference and the pre-exponential factor ratio were calculated.

Most importantly, a comparison of our enzyme-free data to the tunneling data for methylmalonyl-CoA mutase,[2] and other B_{12}-dependent enzymes is now possible, Table 3.1. The KIEs, activation energy difference and ratios of pre-experimental factors are all *the same within experimental error* and if the true error bars of each measurement are taken into account. The only case where it *looks* like there might be a difference beyond experimental error is vs the (small) A_H/A_D ratio for methylmalonyl-CoA mutase. However, our independent linear-regression analysis (section 3.S-2 of the Supporting Information)[58],[59] of that original data provides a slightly different A_H/A_D ratio and significantly larger error bars, $A_H/A_D = 0.082 \pm 0.028$ (vs the author's 0.078 ± 0.009),[2] as well as a somewhat different values for the (E_D-E_H) term, 3.54 ± 0.19 (vs the author's

3.41 ± 0.07).[2] The statistically valid conclusion, then, from the data in Table 3.1 is that the enzymic and enzyme-free tunneling criteria are *the same within experimental error.*

The above findings are important enough and perhaps far-reaching enough that we wanted a second system where we could check our data. In particular, if one can stop Ado• cyclization, Scheme 3.2, then one can measure the Ado-H to Ado-D product ratio directly, pathways 2 and 3, Scheme 3.2. We previously synthesized and characterized 8-MeOAdoCbl[60] for just this purpose, and have demonstrated that 8-MeOAdoCbl thermolysis in ethylene glycol solution yields Co(II)Cbl• and 8-MeOAdo•. As desired, 8-MeOAdo• does *not* cyclize (no path 1, Scheme 3.2) when thermolyzed in ethylene glycol.[60,61] This in turn allowed a more direct determination of the KIE by analysis of the ratio of 8-MeoAdo-H to 8-MeoAdo-D.

As was done previously, 8-MeoAdoCbl was thermolyzed at 10 °C increments from 80 °C to 120 °C, this time in a mixture of 15% ethylene glycol and 85% ethylene glycol-d_4. Conversion to Co(II)Cbl• was again observed by UV-visible spectroscopy, Figure 3.S4, and the products were analyzed using HPLC-MS. The KIE was computed from the observed amounts of 8-MeOAdo-H and 8-MeOAdo-D corresponding, respectively, to the m/z: 282 peak and the m/z: 283 peak in the mass spectra (Figure 3.S6), and using equation 3.2. The 14.46 term in equation 3.2 is the required correction term to account for contributions to the m/z: 283 peak from heavy isotopes (i.e., ^{13}C) in the m/z: 282 peak which corresponds to 8-MeOAdo-H.

$$KIE = \frac{k_{aH}}{k_{aD}} = \frac{\dfrac{\text{m/z : 282 peak}}{\%\text{glycol}_{d-0}}}{\dfrac{\text{m/z : 283 peak} - 14.46}{\%\text{glycol}_{d-4}}}$$

Equation 3.2

Even in 85% ethylene glycol-d_4 the peak in the mass spectrum corresponding to 8-MeOAdo-H is larger than the peak corresponding to 8-MeOAdo-D, Figure 3.S-6. A plot of the observed KIE vs temperature reveals a substantial KIE ranging from = 8.1 ± 0.2 at 120 °C to 12.5 ± 0.9 at 80 °C , Figure 3.3. A plot of ln KIE vs 1/T yields an activation energy difference of (E_D-E_H) = 2.1 ± 0.6 kcal mol^{-1} and A_H/A_D = 0.5 ± 0.4, Figure 3.2. The higher error bars intrinsic to the HPLC-MS method[62-66] required to analyze this system are reflected in the resultant 8-MeOAdoCbl data set and its greater error bars. Nevertheless, the data obtained for 8-MeOAdoCbl *are within experimental error of the values obtained for AdoCb*l Figure 3.2 and Table 3.1. The results are again indicative of substantial QM tunneling in the enzyme-free, 8-MeOAdo• H-atom abstraction reaction *as well as no difference within experimental error vs the tunneling data for the enzyme, methylmalonyl-CoA mutase,* Table 3.1.

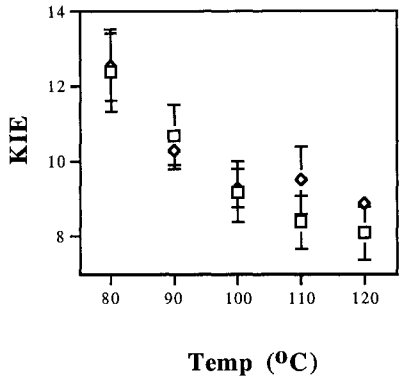


Figure 3.2 A plot of the observed kinetic isotope effects (KIEs) of the hydrogen abstraction from ethylene glycol vs temperature: Ado• (□), 8-MeOAdo• (◇). The results show that both sets of data are identical within experimental error.

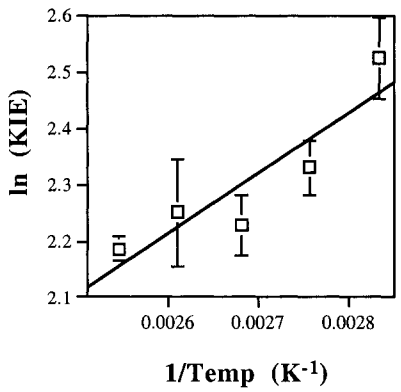


Figure 3.3 A plot of ln KIE vs 1/T for the 8-MeOAdoCbl thermolysis. From the slope and intercept, the activation energy difference and the pre-exponential factor ratio were obtained, (E_D-E_H) of 2.1 ± 0.6 kcal mol^{-1} and A_H/A_D= 0.5 ± 0.4.

Table 3.1 The observed KIE, activation energy difference, and pre-exponential factor ratio for our non-enzyme systems and literature enzyme systems.

	KIE	E_D-E_H (kcal mol^{-1})	A_H/A_D
AdoCbl Solution	12.4 ± 1.1 at 80°C ~29.3 at 20 °C [a] ~35.2 at 10 °C [a]	3.0 ± 0.3	0.16 ± 0.07
8-MeOAdoCbl Solution	12.5 ± 0.9 at 80°C ~21.8 at 20 °C [a] ~24.8 at 10 °C [a]	2.1 ± 0.6 [b]	0.5 ± 0.4 [b]
Methylmalonyl-CoA mutase[2]	35.6 at 20 °C ~12.7 at 80 °C [c]	3.41±0.07 (3.54 ± 0.19) [d]	0.078±0.009 (0.082 ± 0.28) [d]
Ethanolamine ammonia lyase[67,68]		(3.1 ± 1.1) [e]	(0.038 ± 2.13) [e]
Glutamate mutase[69,70]	28 to 35 at 10 °C	NA [f]	NA [f]
diol dehydratase[33,71-73]	8 and 28.6 at 10 °C	NA [f]	NA [f]
GS-ZPE 1° only	6.4 at 20 °C 6.8 at 10 °C	1.2	1.0
GS-ZPE 1° x 2°	8.9 at 20 °C [g] 9.5 at 20 °C [g]	1.2	1.0

[a] Values are extrapolated from the higher temperature data and represent lower limits to the true values.
[b] The larger error bars for this 8-MeOAdoCbl data set are due to the intrinsically larger errors of the ion-trap HPLC-MS method used to analyze the products.[62-66]
[c] This 80 °C value was obtained by extrapolation from the lower temperature data set.[2]
[d] These are our linear-regression analysis of the literature data set.[2] See the Supporting Information, section 3.S-2, for further details.
[e] These data were calculated from data in the literature.[68] See the Supporting Information, section 3.S-4 for further details.
[f] NA = not available in the literature.
[g] The GS-ZPE 1^{o} and 2^{o} KIE = $[(1^{o}\text{KIE}) \times 1.15 \times 1.1^{2}]$.

The above data complete the first experimental test of the hypothesis of enzyme-enhanced tunneling. They reveal that, at least in the H• abstraction reaction by Ado•, the enzymes have *not* evolved to enhance QM tunneling. Instead, they simply exploit the identical level of QM tunneling available in the enzyme-free, solution reaction.

Two caveats merit mention in closing. First, as already noted the H-atom abstraction reactions from ethylene glycol and methylmalonyl-CoA are not *identical* reactions, although they do involve reactions with very close bond energies; little difference in their degree of tunneling is expected.[31,32,38-40] Indeed, ex post facto support for the comparison and the assumption that the systems will not have substrate-dependent tunneling differences is provided by fact that the data sets are the same within experimental error and by the close similarity between the proposed diol dehydratase mechanism[74] and the methylmalonyl-CoA mutase mechanism.[75] A second caveat is that our enzyme-free reactions were, necessarily, studied at a higher temperature range than the enzymic systems. Again, the consistency of the data suggest that this is not an issue as does our finding of identical results within experimental error for a β-neopentylCbl system which undergoes the needed Co-C thermolysis reaction in the same temperature range as the enzyme.[57,58]

That system also addresses the issue of whether or not Ado• is special in its degree of tunneling vs a simple alkyl R•—so far, the answer is that Ado• is *not* special.[57,58]

Finally, although this first experimental test of Klinman's hypothesis strongly suggests that enzymes have not evolved to enhance QM tunneling, more studies are needed of different enzymes using the method of Scheme 3.1 plus the Kreevoy criteria to know whether of not the "no-enhancement" results herein are more generally applicable. It is *possible* that these results from B_{12}-enzymes are a special case: the B_{12} cofactor itself may be of prebiotic origins,[76,77] and many B_{12} enzymes are ancient systems.[76,78,79] In addition, there appears to have been little evolutionary pressure to enhance the H• abstraction values in these enzymes past their bimolecular solution values of ca. 7×10^3 M^{-1} sec^{-1} [47,80] since these rates are already fast enough for the fairly slow[81,82-84] turnover rates for B_{12}-dependent enzymes, ca. 22-600 sec^{-1}.[75,85] Nevertheless, the starting hypothesis for future work now has to be the main finding from this work: that there is no experimentally documented case where an enzyme has evolved to enhance QM tunneling, and there is one case –the present work– where the data unequivocally shows that there is no enhancement of QM tunneling within experimental error. Instead, the B_{12}-dependent enzyme methylmalonyl-CoA mutase simply exploits the *same* level of tunneling available in the enzyme-free, solution, Ado• abstraction reaction. In fact, the results strongly suggest an unchanged barrier shape (i.e., both width and height) for both the enzyme and enzyme-free reactions. The present work also illustrates the importance of chemical precedent studies, especially when those studies can be done with the exact cofactor in an enzyme-free system.[48]

Experimental

Materials

The following were used as received: adenosylcobalamin (AdoCbl; Sigma, 98%), adenosine (Sigma), methanol (Fisher Scientific, HPLC grade), acetonitrile (Fisher Scientific, HPLC grade), argon (General Air), water (Fisher Scientific, HPLC grade (for use in the HPLC-MS)), ethylene glycol-d_6 (Cambridge Isotope Labs, 98%). The chemical and isotopic purities of ethylene glycol-d_0 (Aldrich, 99.8% anhydrous) and ethylene glycol-d_4 (Cambridge Isotope Labs, 98%) were confirmed by GC-MS, hence these were used as received. Distilled water was filtered through a Barnstead nano-pure filtration system. 8-methoxy-5'-deoxycobalamin (8-MeOAdoCbl) was synthesized according to a literature procedure.[60]

Instrumentation and Equipment

UV-visible absorption spectra (± 1 nm) were recorded on a Hewlett-Packard Model 8452A UV-visible 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 x 4.6 mm Alltech C-18 reverse phase column. HPLC-MS was done with an HP 1100 HPLC with a 200 mm x 4.6 mm microsorb C-18 column coupled to a Finnigan LCQ Duo mass spectrometer. The HPLC-MS was set to an HPLC flow rate of 0.7 mL/min, a capillary temperature of 250 °C, and the positive ion mode was selected with a mass window of 100-1650 m/z. ^{1}H NMR spectra were recorded on an Inova-300 spectrometer operating at room temperature and were referenced internally to 0 ppm with TSP (D_2O). All linear regressions were performed on a Power Macintosh 5400/120 using Microsoft Excel 98, except the

independent regression analysis on the methylmalonyl-CoA data (Section 3.S-2) which was performed a total of three times using three different software packages on two different computers. Origin 3.5 on an MCW 486 personal computer running the Windows 3.1 operating system; and Kaleidagraph 3.51, and Microsoft Excel 98, both on a Power Macintosh 5400/120.

The thermolyses of AdoCbl and 8-MeOAdo were carried out in Schlenk cuvettes[47] prepared by glass blowing PTFE needle valves onto 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 AdoCbl in ethylene glycol). No detectable decomposition was observed over the time scale used in our thermolyses (~1 week).

Thermolysis temperatures were maintained by immersing the cuvettes in a 2 L oil-bath equipped with a magnetic stir bar and a wound wire-heating element attached to a Barnant temperature controller. The temperature was verified (± 1 ^{o}C) using a mercury thermometer with gradations covering the temperature range of interest.

All samples were prepared in a Vacuum Atmospheres inert atmosphere drybox. The O_2 level of < 2 ppm was monitored by a Vacuum Atmospheres model AO 316-C oxygen analyzer.

Adenosylcobalamins are very 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 Adenosylcobalamin Thermolyses and Analysis Procedure

First, ~1.0 mg of AdoCbl was weighed into a foil wrapped vial and taken into the drybox. Inside the drybox, 3.0 mL of ethylene glycol (degassed 3 times by freeze/evacuate/refill with argon/thaw cycles) was added with a syringe giving a ~2 x 10^{-4} M solution. The solution was transferred into foil-wrapped Schlenk cuvettes. Samples analyzed by HPLC were not diluted, whereas samples for UV-visible spectroscopy were diluted with ethylene glycol-d_0 to ~ 7 x 10^{-5} M. The cuvettes were brought out of the drybox and into the darkroom for thermolysis. Four to thirteen thermolyses (see Table 3.S1) in both ethylene glycol-d_0 and ethylene glycol-d_4 were carried out at temperatures of 80 °C, 90 °C, 100 °C, 110 °C, and 120 °C for ≥ 6 half lives,[47] corresponding to at least 98.5% conversion as confirmed by UV-visible spectra taken before and after thermolysis. For one of the 90 °C experiments, UV-visible spectra were taken at regular intervals (Figure 3.S1) and clean isosbestic points at 336 nm, 394 nm, 486 nm, and 586 nm were observed. The spectra indicate direct and complete conversion to Co(II)Cbl$^{\bullet}$.[47] The ~2.4 x 10^{-4} M solutions were analyzed by HPLC (instrumentation described above; isocratic, 95% H_2O/ 5% CH_3CN, 2 mL/min, monitored at 254 nm; for typical HPLC traces in ethylene glycol-d_0 and ethylene glycol-d_4 see Figures 3.S2 and 3.S3). The HPLC chromatograms for an ethylene glycol-d_0 reaction show a small adenine peak (retention time ~ 4.8 min, ~ 4% total peak area), a larger peak corresponding to 3,5'-anhydroadenosine (c-Ado; retention time ~8.9min, ~ 66% total peak area), and a peak corresponding to 5'-deoxyadenosine (Ado-H; retention time ~13.1 min, ~ 24% total peak area). The HPLC chromatograms for an ethylene glycol-d_4 reaction show a small adenine peak (retention time ~ 4.8 min, ~ 7% total peak area), a larger peak corresponding to 3,5'-anhydroadenosine (c-Ado; retention time ~8.5min, ~ 79% total

peak area), and a peak corresponding to 5'-deoxyadenosine (Ado-D; retention time ~12.5 min, ~ 2.3% total peak area).[86] The peaks were assigned by comparison of retention times for authentic materials.[47] The ratio of the [(c-Ado/Ado-H)/(c-Ado/Ado-D)] gives the KIE (derivations are available in section 3.S-6 in the Supporting Information).

The 8-methoxy-5'-deoxy-adenosylcobalamin Thermolyses and Analysis Procedure

First, 5.0 mg (2.8×10^{-6} mol corrected for 10 waters of hydration) of 8-MeOAdoCbl was weighed out inside a foil wrapped vial and brought into the drybox. Inside the drybox, 0.7 mL of ethylene glycol-d_0 (degassed 3 times by freeze/evacuate/refill with argon/thaw cycles) was added to the vial. The Schlenk cuvettes were filled with either 0.75mL or 0.85 mL ethylene glycol-d_4 (also degassed) and degassed ethylene glycol-d_0 was added to bring the volume up to 0.9 mL. Next, 0.1 mL of the 8-MeOAdoCbl solution was added to each vial, resulting in a ~ 4×10^{-4} M solution (a lower concentration ~1.2×10^{-4} M, was tried, but the HPLC-MS signal was substantially better using the 3 fold higher concentration) in which the solvent was either 75% or 85% ethylene glycol-d_4 and the remaining solvent was ethylene glycol-d_0. The vials were covered with aluminum foil. In a Schlenk cuvette, another 0.1 mL portion was diluted to 3.0 mL with ethylene glycol-d_0 and then covered with aluminum foil. This cuvette was used for the UV-visible spectroscopic analysis. All of the cuvettes were brought out of the drybox and into the darkroom for thermolysis. Thermolysis reactions were run for ≥ 6 half lives at 80 °C (154 hours), 90 °C (105 hours, 40 min), 100 °C (33 hours), 110 °C (21 hours), and 120 °C (4 hours, 48min). UV-visible spectra were taken at regular intervals, Figure 3.S4. Isosbestic points at 338 nm, 392nm, 486nm, and 585nm

were observed indicating complete conversion to Co(II)Cbl•. The samples were injected into the HPLC-MS (HPLC flow rate 0.7 mL/min, H_2O/MeOH 90%/10% ramped to 5%/95% over 55 min). HPLC traces were monitored at 256 nm, and the mass spectrum was recorded over a 100-1650 m/z ratio.[87] Peaks were identified by the molecular ion peaks in their mass spectrum. A typical HPLC trace (Figure 3.S5), had an early small peak (~7 min) identified as 8-MeO-adenine, a large later peak (~13 min) identified as 8-methoxy-5'-deoxy adenosine and a later peak (~22 min) that was identified as a cobalamin species. The KIE was from the ions with an m/z ratio of 281-284, Figure 3.S6 and using equation 3.2. Because the m/z ratio of 282 corresponds to [8-MeOAdo-H+H^+]$^+$ and the m/z ratio of 283 corresponds to [8-MeOAdo-D+H^+]$^+$, the KIE can be calculated from the m/z: 282 peak and the m/z: 283 peaks. The m/z: 283 peak must be corrected for contribution from heavier isotopes in the non-deuterated product, a correction term of 14.46 was calculated using the software package on a Fisions Quatro mass spectrometer, Mass Lynx. The KIE was then calculated by taking the ratio of the m/z: 282 peak over ethylene glycol-d_0 as the numerator, divided by the corrected m/z: 283 peak over ethylene glycol-d_4 as the denominator, Equation 3.2.

Supporting Information

Supporting information is available as usual from the ACS web site as well as in the Ph. D. thesis of K. M. Doll (Colorado State University Spring 2003): Section 3.S-1: A Control Experiments Using Ethylene Glycol-d_6, Adenosine Control Experiment Demonstrating that Possible Proton Exchange is not Affecting the Observed Products, The choice of HPLC-MS over Direct Injection MS. Section 3.S-2: Discussion and Plot of ln KIE vs 1/T using the data from Banerjee. Section 3.S-3: Discussion of Tunneling Evidence in Diol Dehydratase. Section 3.S-4: Discussion of Ethanolamine Ammonia Lyase including a Plot of ln k vs 1/T for Both Protiated and Deuterated Substrates. Section 3.S-5: A Short Discussion of Tunneling Evidence in Glutamate Mutase. Section 3.S-6: Derivation of Equation 3.1. Section 3.S-7 Relation of E_D-E_H and A_H/A_D to a ln KIE vs 1/T plot. Figure 3.S1: The UV-visible spectra of an AdoCbl thermolysis reaction hrs at 90 °C. Figure 3.S2: An HPLC chromatogram for an ethylene glycol-d_0 reaction at 80 °C. Figure 3.S3: An HPLC chromatogram for an ethylene glycol-d_4 reaction at 80 °C. Figure 3.S4: The UV-visible spectra of an 8-MeOAdoCbl thermolysis reaction hrs at 90 °C; Figure 3.S5: The representative HPLC-MS from a 100 °C thermolysis reaction of 8-MeOAdoCbl in 85% ethylene glycol-d_4. Figure 3.S6 A representative comparison of the corresponding ratios of 8-MeOAdo-H (281.9) and 8-MeOAdo-D (282.9). Table 3.S1: The ratio c-Ado/Ado-H in post-thermolysis Ado-B_{12} in ethylene glycol-d_0. Table 3.S2: The ratio c-Ado/Ado-H in post-thermolysis Ado-B_{12} in ethylene glycol-d_0. This material is available free of charge via the Internet at http://pubs.acs.org.

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(87) Since it is possible that the m/z window setting on the mass spectrometer could have affected the observed product ratios by changing the number of ions captured in the ion trap, a control experiment varying the m/z window setting was performed. An 8-MeOAdoCbl thermolysis solution was analyzed by MS-HPLC with the 100-1650 m/z window and then immediately reanalyzed with the MS-HPLC set to monitor only a 270-300 m/z window. The results were the same within experimental error.

Supporting Information

The First *Experimental* Test of the Hypothesis that Enzymes Have Evolved to Enhance Quantum Mechanical Tunneling

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Section 3.S-1

A Control Experiment Using Ethylene Glycol-d_6

A control experiment was performed using ethylene glycol-d_6, in order to test for the possibility of hydrogen abstraction from the oxygen atoms of the ethylene glycol. A 110 °C thermolysis was performed, using the same procedure and HPLC analysis as in the ethylene glycol-d_4 case. The KIE was found to be 8.7 ± 0.3. This is within the experimental error of our previous 110 °C result. In addition, an ethylene glycol-d_4 experiment was performed at the same time, showing a KIE of 8.7 ± 0.1.

Adenosine Control Experiment Demonstrating that Possible Proton Exchange is not Affecting the Observed Products.

In an important control experiment, a 6.4 x 10^{-4} M solution of authentic adenosine in ethylene glycol at the highest level of deuteration used in our experiments (85% ethylene glycol-d_4, 15% ethylene glycol-d_0) was thermolyzed under exactly the same conditions as the 110 °C thermolysis reaction of 8-MeOAdoCbl. The solution was subjected to HPLC-MS also under the exact same conditions as an 8-MeOAdoCbl thermolysis sample (HP 1100 HPLC with a 200 mm x 4.6 mm microsorb C-18 column coupled to a Finnigan LCQ Duo mass spectrometer, HPLC flow rate 0.7 mL/min, H_2O/MeOH 90%/10% ramped to 5%/95% over 55 min). The ratio of the m, m+1, m+2, m+3 peaks (m/z 268, 269, 270, 271) were all within 2% of the expected values for adenosine, calculated by the isotopic simulator software program of the Fissions Quatro mass spectrometer, Mass Lynx. This experiment demonstrates either that any

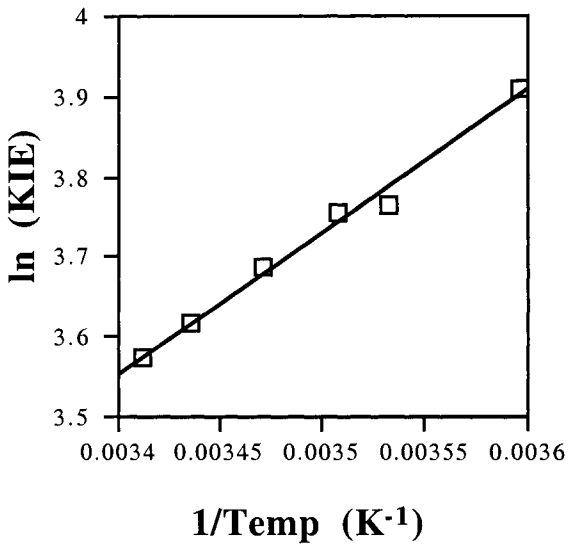
exchangeable hydrogens are not exchanging with deuterium in the solvent, or that they have exchanged back to their protiated form under our analysis conditions.

The choice of HPLC-MS over Direct Injection MS.

We attempted to analyze several of the (110 °C) 8-MeOAdoCbl thermolysis solutions by simple dilution and directly injecting them into the same Finnigan LCQ Duo mass spectrometer. The results were irreproducible and did not give a clean mass signal in the region of interest (m/z 282-283). We abandoned this method in favor of the superior HPLC-MS method.

Section 3.S-2 Discussion and Plot of ln KIE vs 1/T Using the Data From Banerjee

Data from table 1 in the Banerjee[1] paper was plotted and a linear regression was performed using three different software packages on two different computers: Origin 3.5 on an MCW 486 personal computer running the Windows 3.1 operating system, and Kaleidagraph 3.51 and Microsoft Excel 98, both on a Power Macintosh 5400/120. The linear regression gives a slope of 1779.6 ± 96.4 and an intercept of -2.497 ± 0.336 in each case. Calculations using this slope and intercept with error propagation[2,3] give an activation energy difference (E_D-E_H) of 3.54 ± 0.19 kcal mol^{-1} and a pre-exponential factor ratio of (A_H/A_D) of 0.082 ± 0.028. These values are similar to the values of Banerjee and co-workers of (E_D-E_H) of 3.41 ± 0.07 kcal mol^{-1} and (A_H/A_D) of 0.078 ± 0.009, however, our error bars are larger. Two possible reasons for the discrepancy are: the addition of two higher temperature data points shown the Figure 2-inset elsewhere,[1] but not given in Table 1 elsewhere,[1] and hence that we could not use, and the omission of two data points in the 5-20 °C range in the Figure 2-inset given elsewhere.[1]



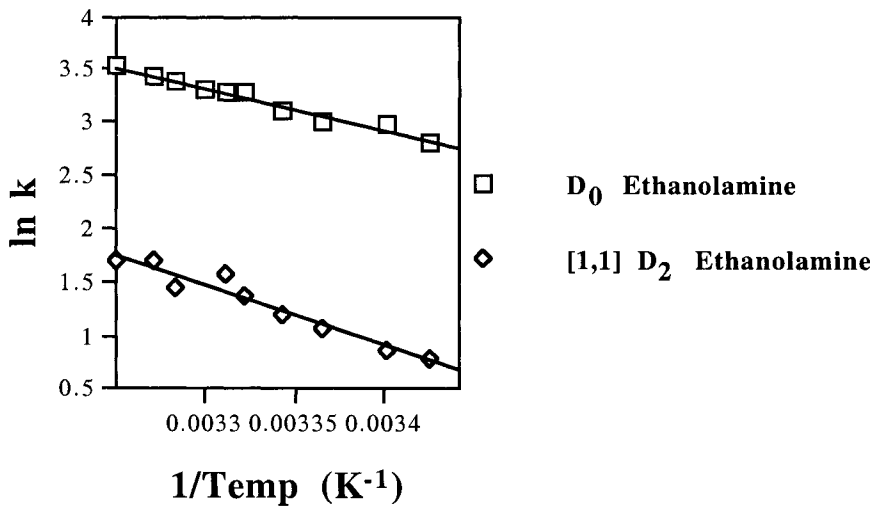
A plot of ln KIE vs 1/T using the data from Banerjee's[1] table 1.

Section 3.S-3 Discussion of Tunneling Evidence in Diol Dehydratase

Diol dehydratase is an enzyme which converts vicinal diols (including ethylene glycol) into the corresponding aldehydes and water.[4,5] As in methylmalonyl-CoA mutase, the Co–C bond is cleaved, generating Co(II)Cbl$^{\bullet}$ and at least a formal Ado$^{\bullet}$, and the Ado$^{\bullet}$ abstracts a hydrogen from the substrate. This is followed by a novel hydroxyl radical migration which Toraya[6] postulates (and Radom calculates[7]) is assisted by a potassium cation located in the active site of the enzyme. A hydrogen is then reabstracted from Ado-H by the substrate radical, now a gem-diol radical.[6] This back abstraction causes difficulty in interpreting the results since the Ado-H(D) reabstraction reaction also has an isotope effect. The first step appears to show a k_H/k_T isotope effect of 20 while the second step shows a very large k_H/k_T isotope effect of 125 at 10 °C[8] corresponding to k_H/k_D values of 8.0 and 28.6, respectively,[9] using the Swain-Schaad equation.[10] However, other workers have found smaller deuterium KIEs (ca. 2 at 37 °C) for H vs. D-atom abstraction from substrate for diol dehydratase by deriving them from kinetic fits to observed substrate turnover rate constants for various deuterated substrates.[11-13] It has been noted by Cleland that at least two different H-atom transfer pathways are required to explain all of the data.[14] The interpretation for tunneling here is not as definitive as the methylmalonyl-CoA mutase system, however, as discussed in a general way by Northrop,[15] Klinman,[16] and Sutcliffe,[17] and even more specifically for diol dehydratase[18] by Richards,[12] such kinetic complexity may be masking the evidence for tunneling.

Section 3.S-4 Discussion of Ethanolamine Ammonia Lyase including a Plot of ln k vs 1/T for Both Protiated and Deuterated Substrate

A plot of ln k vs 1/T for both the protiated and deuterated substrates from Babior's work and Figure 4 elsewhere[19] was made. The slope and intercept on the ethanolamine-d_0 data are 4071 ± 224 and 16.8 ± 0.7. The slope and intercept on the ethanolamine-d_2 data are 5623 ± 518 and 20 ± 2.0. This yields the following activation energies and pre-exponential factors: E_{D0}=8.1 ± 0.4 kcal mol^{-1}, E_{D2}=11.2 ± 1.0 kcal mol^{-1}, a difference of 3.1 ± 1.1 kcal mol^{-1}. A_{d0}=1.90 ± 1.40 x 10^7 and A_{d2}= 4.9 ± 9.7 x 10^8, a ratio A_H/A_D= 0.038 ± 2.13. Both of these criteria are within the range indicative of tunneling, although the large error on the A_H/A_D ratio makes this criterion non-definitive.



The KIE for the isotope effect on the formation of Co(II)Cbl• has also been studied recently (KIE > 10), and the possibility of quantum-mechanical tunneling has been suggested by the authors.[20]

Section 3.S-5 A Short Discussion of Tunneling Evidence in Glutamate Mutase

Large deuterium KIEs in glutamate mutase have been observed by Marsh et al.[21,22] The KIEs ranged from 28 to 35 at 10 °C depending on substrate. Marsh has also measured a $k_H/k_T > 100$,[23] raising the possibility that this enzyme also uses quantum-mechanical tunneling.[24]

Section 3.S-6

Derivation of Equation 1

Assumptions

Several assumptions are necessary in order to derive Equation 1: (1) The cyclization step in Scheme 3.2 (in the main text) is irreversible at all the temperatures examined (80-120 °C), and the rate of formation of c-Ado depends only on k_c. In other words, all steps leading to product after k_c are fast; (2) The formation of c-Ado and Ado-H is kinetically controlled. That is, c-Ado and Ado-H are stable under the conditions studied. This was verified experimentally (vida infra); (3) The "hydrogen" radical lost from cyclic-Ado• (e.g., possibly as H-Cbl from the reaction of the cyclic-Ado$^{\bullet}$ with Co(II)Cbl$^{\bullet}$) and the ethylene glycol radical generated during the course of reaction do not influence any other steps in Scheme 3.2 in the main text; and (4) the hydrogen abstracted from ethylene glycol or ethylene glycol-d_4 is from the carbon, and not from the oxygen. This is expected from the literature (for methyl radical abstractions from MeOH)[25,26] and was confirmed by a control thermolysis (Section 3.S-1) using ethylene glycol-d_6 which showed identical results as seem with ethylene glycol-d_4.

Justification for Assumptions

(1) The cyclization step in Scheme 3.2 (in the main text) with rate constant k_c is irreversible and the rate of formation of c-Ado depends only on k_c:

This assumption is reasonable because (a) the 5'-deoxyadenosine radical is a primary alkyl radical while that of the cyclized product is a secondary, nitrogen-based "benzylic-like" radical. If Ado$^{\bullet}$ and the cyclized product radical, c-Ado$^{\bullet}$ were in equilibrium, the equilibrium (enthalpy) should favor the more stable cyclized radical in any event.

(b) Even if AdoCbl, Ado$^{\bullet}$, and c-Ado$^{\bullet}$ were in rapid equilibrium, all of these equilibria are independent of glycol isotopic substitution and *should not be isotope sensitive.* To a first approximation, only the H (D) atom abstraction step from solvent should be isotope

sensitive (Derivation 2), and that step is very likely to be irreversible, given the overwhelming presence of the more reactive glycol.

(2) The c-Ado /Ado-H ratio is kinetically controlled: Both c-Ado and AdoH are stable under the conditions studied. 8, 5'-anhydroadenosine (c-Ado) and 5'-deoxyadenosine were heated together with hydroxocobalamin (which gives Co(II)Cbl$^{\bullet}$ on heating) in ethylene glycol under anaerobic conditions at 110 °C and 90 °C for 9.6 hr and 94.8 hr respectively. HPLC showed no changed in the c-Ado/AdoH ratios. AdoCbl solutions that were deliberately exposed to various amounts of room light and low levels of air before thermolysis showed no substantial difference in c-Ado/Ado-H ratio from those carefully safeguarded from these adventitious factors.

(3) The "hydrogen" radical lost from cyclic-Ado• (e.g., probably as H–Cbl from the reaction of the cyclic-Ado• with Co(II)Cbl$^{\bullet}$) and the ethylene glycol radical generated during the course of reaction do not influence any other steps in Scheme 3.2:

The most probable fate of the nucleoside weak H-atom radical and the ethylene glycol radical is their reaction with the persistent radical, Co(II)Cbl$^{\bullet}$.[27] There is no evidence for the ethylene glycol radical participating in any of the three pathways in Scheme 3.2.

We have also considered the isotope effect on solvent density and viscosity for ethylene glycol and ethylene glycol-d_4 and, thereby their possible additional effects on the observed KIE. While we found no literature data for viscosity and molar volume changes for deutero ethylene glycol, literature data for other simple liquids, H_2O/D_2O,[28] dmso/dmso-d_6 , methanol/methanol-d_4 indicates that deuteration changes both molar volumes (deuterated solvents are slightly denser) and viscosities (deuterated solvents are usually more viscous). The slight increased density of deuterated solvents implies a slightly higher molar activity for ethylene glycol-d_4 (effective concentration). However, this trend should lead to a decrease in KIE, via the effect on the rate of D-atom abstraction. On the other hand, for pure hydrocarbons like heptane, the effect of deuteration is to decrease viscosity[29] (perdeuteration is equivalent to an 8 °C increase in temperature). Without accurate data on relative viscosities of ethylene glycol and

ethylene glycol-d_4, meaningful differences remain unclear. In any case, however *the difference should be significantly smaller that the experimental error in our system.*

Derivation 1: Assuming Irreversible Cyclization

The rates of formation of c-Ado and Ado-H are expressed as:

$$d[\text{c-Ado}]/dt = k_c[\text{Ado}^\bullet] \quad \text{(S1)}$$

$$d[\text{Ado-H}]/dt = k_a\,[\text{Ado}^\bullet][\text{EG}] \quad \text{(S2)}$$

Integrating both sides of S1 and S2,

$$[\text{c-Ado}] = k_c \int [\text{Ado}^\bullet]\, dt \quad \text{(S3)}$$

$$[\text{Ado-H}] = k_a\,[\text{EG}] \int [\text{Ado}^\bullet]\, dt \quad \text{(S4)}$$

Dividing S3 by S4,

$$[\text{c-Ado}]/[\text{Ado-H}] = k_c/(k_a[\text{EG}]) \quad \text{(S5)}$$

Using [EG] and [EG-$_{d4}$] to denote ethylene glycol-d_0 and ethylene glycol-d_4, Equation S5 becomes:

$$[\text{c-Ado}]/[\text{Ado-H}] = k_c/(k_{aH}\,[\text{EG}]) \quad \text{(S6)}$$

$$[\text{c-Ado}]/[\text{Ado-D}] = k_c/(k_{aD}\,[\text{EG-}d_4]) \quad \text{(S7)}$$

The concentration ratio [c-Ado]/[Ado-H] is directly proportional to the peak area ratio, $A_{\text{c-Ado}}/A_{\text{Ado-H}}$; these ratios can be scaled by a response factor, S:

$$[\text{c-Ado}]/[\text{Ado-H}] = A_{\text{c-Ado}}/A_{\text{Ado-H}}) \times S = r(H) \times S \quad \text{(S8)}$$

$$[\text{c-Ado}]/[\text{Ado-D}] = A_{\text{c-Ado}}/A_{\text{Ado-D}}) \times S = r(D) \times S \quad \text{(S9)}$$

Substituting, equations 6 and 7 become

$$k_c/(k_{aH} \times [\text{EG}]) = r_H \times S \quad \text{(S10)}$$

$$k_c/(k_{aD} \times [\text{EG}]) = r_D \times S \quad \text{(S11)}$$

Dividing S11 by S10,

$$k_{aH}/k_{aD} = r(D)/r(H) \quad \text{(S12)}$$

Equation S12 is equivalent to equation 1 in the text and is the basis for measurement of the kinetic isotope effect (KIE) on H vs D atom abstraction from ethylene glycol solvent.

Derivation 2: Assuming Rapid Equilibrium

Consider the case when Ado• and c-Ado• are in rapid equilibrium:

$$\text{AdoH} \xleftarrow{k_a\,\text{SH}} \left\{ \text{Ado}^\bullet \rightleftharpoons \text{c-Ado}^\bullet \right\} \xrightarrow{k_{\text{H-}}} \text{c-Ado} + \text{H}^\bullet$$

$$\frac{d[\text{AdoH}]}{dt} = k_a\,[\text{Ado}^\bullet]\,[\text{SH}] \qquad (S_2 1)$$

$$\frac{d[\text{c-Ado}]}{dt} = k_{\text{H-}}\,[\text{c-Ado}^\bullet] \qquad (S_2 2)$$

Because of the rapid equilibrium, we cannot distinguish Ado• from c-Ado•, the two species in the curly bracket above.

$$\text{Let } [\text{Ado}]_T = [\text{Ado}^\bullet] + [\text{c-Ado}^\bullet] \qquad (S_2 3)$$

$$\text{Using } K_C \text{ as defined above, } [\text{Ado}^\bullet] = \frac{[\text{Ado}]_T}{1 + K_c} \qquad (S_2 4)$$

$$\text{So } \frac{d[\text{AdoH}]}{dt} = \frac{k_a\,[\text{SH}]\,[\text{Ado}]_T}{1 + K_c} \qquad (S_2 5)$$

likewise, solving equation ($S_2$2) in terms of $[\text{Ado}]_T$:

$$[\text{c-Ado}\bullet] = \frac{K_C\,[\text{Ado}]_T}{1 + K_c} \qquad (S_2 6)$$

$$\frac{d[\text{c-Ado}]}{dt} = k_{\text{H-}}\,\frac{K_C\,[\text{Ado}]_T}{1 + K_c} \qquad (S_2 7)$$

The ratio r(H) is equation ($S_2$5)/equation ($S_2$7) is equation ($S_2$8):

$$\frac{\text{Eq 5}}{\text{Eq 7}} = \frac{\dfrac{d[\text{AdoH}]}{dt}}{\dfrac{d[\text{c-Ado}]}{dt}} = \frac{k_{\text{H-}}\,\dfrac{K_C\,[\text{Ado}]_T}{1 + K_c}}{\dfrac{k_a\,[\text{SH}]\,[\text{Ado}]_T}{1 + K_c}} = \frac{k_{\text{H-}}\,K_C}{k_a\,[\text{SH}]} = \text{r(H)} \qquad (S_2 8)$$

A corresponding set of equations could be written for the EG-d_4 case, r(D):

$$\frac{\dfrac{d[\text{AdoD}]}{dt}}{\dfrac{d[\text{c-Ado}]}{dt}} = \frac{k_{\text{H-}}\,\dfrac{K_C\,[\text{Ado}]_T}{1 + K_c}}{\dfrac{k_{a(D)}\,[\text{SD}]\,[\text{Ado}]_T}{1 + K_c}} = \frac{k_{\text{H-}}\,K_C}{k_{a(D)}\,[\text{SD}]} = \text{r(D)} \qquad (S_2 9)$$

Dividing equation ($S_2$8) by ($S_2$9) gives the equivalent of equation S12 in derivation1

$$r(D)/r(H) = k_a/k_{a(D)}$$

Section 3.S-7 Relation of E_D-E_H and A_H/A_D to a ln KIE vs 1/T plot

The definition of the deuterium kinetic isotope effect:

$$(1) \quad KIE = \frac{k_H}{k_D}$$

The appropriate Arrhenius equations:

$$(2) \quad k_H = A_H \times e^{\left(\frac{-E_H}{RT}\right)} \text{ and } k_D = A_D \times e^{\left(\frac{-E_D}{RT}\right)}$$

Substitution of (2) into (1) gives:

$$(3) \quad KIE = \frac{A_H \times e^{\left(\frac{-E_H}{RT}\right)}}{A_D \times e^{\left(\frac{-E_D}{RT}\right)}}$$

Rearranging

$$(4) \quad KIE = \frac{A_H}{A_D} \times e^{\left(\frac{E_D - E_H}{RT}\right)}$$

Taking the ln of both sides:

$$(5) \quad \ln KIE = \ln\left(\frac{A_H}{A_D}\right) + \left(\frac{E_D - E_H}{RT}\right)$$

Rearranging:

$$(6) \quad \ln KIE = \ln\left(\frac{A_H}{A_D}\right) + \left(\frac{1}{T}\right)\left(\frac{E_D - E_H}{R}\right)$$

So a plot of ln KIE vs 1/T will have an intercept of ln (A_H/A_D) and a slope of (E_D-E_H)/R.

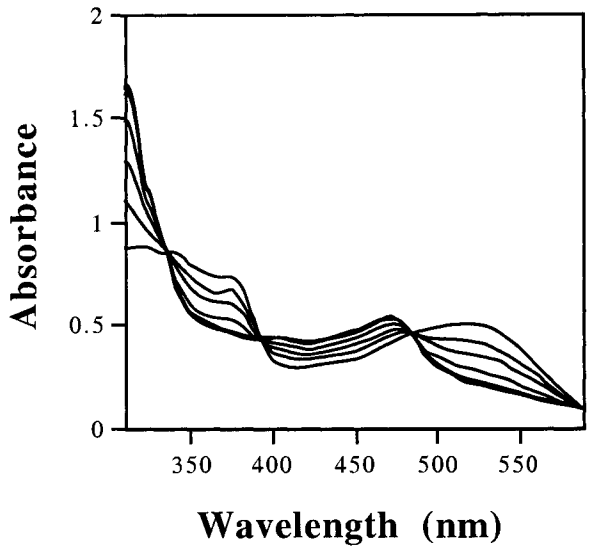


Figure 3.S1 The UV-visible spectra of an AdoCbl thermolysis reaction at 90 °C at 0, 7.00, 12.58, 25.25, 45.58, 73.92, and 92.63 hours. Isosbestic points are observed at 336, 394, 486, and 586 nm indicating clean conversion to Co(II)Cbl•.

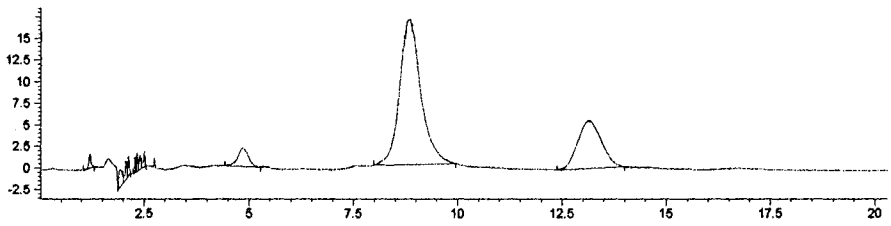


Figure 3.S2 An HPLC chromatogram for an ethylene glycol-d_0 reaction at 80°C. A small adenine peak (retention time ~ 4.8 min, ~ 4% total peak area), a larger peak corresponding to 3,5'-anhydroadenosine (c-Ado; retention time ~ 8.9min, ~ 66% total peak area), and a peak corresponding to 5'-deoxyadenosine (Ado-H; retention time ~ 13.1 min, ~ 24% total peak area) are all present. In two of the HPLC chromatograms, there was a small peak, at ~7.7 min, ~8% total peak area. Because this unidentified peak was only present in two samples, and since there was no corresponding peak in the ethylene glycol-d_4 system, this peak was used in the KIE calculations.

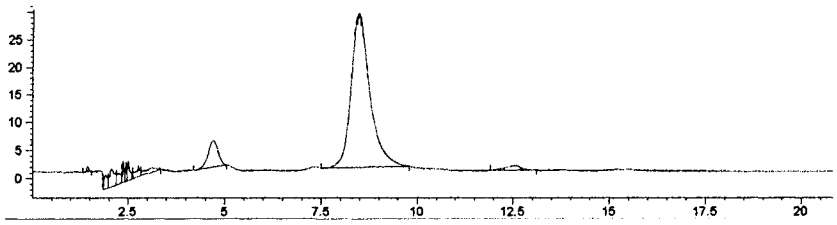


Figure 3.S3 An HPLC chromatogram for an ethylene glycol-d_4 reaction at 80°C. A small adenine peak (retention time ~ 4.8 min, ~ 7% total peak area), a larger peak corresponding to 3,5'-anhydroadenosine (c-Ado; retention time ~ 8.5min, ~ 79% total peak area), and a peak corresponding to 5'-deoxyadenosine (Ado-D; retention time ~ 12.5 min, ~ 2.3% total peak area) are all present.

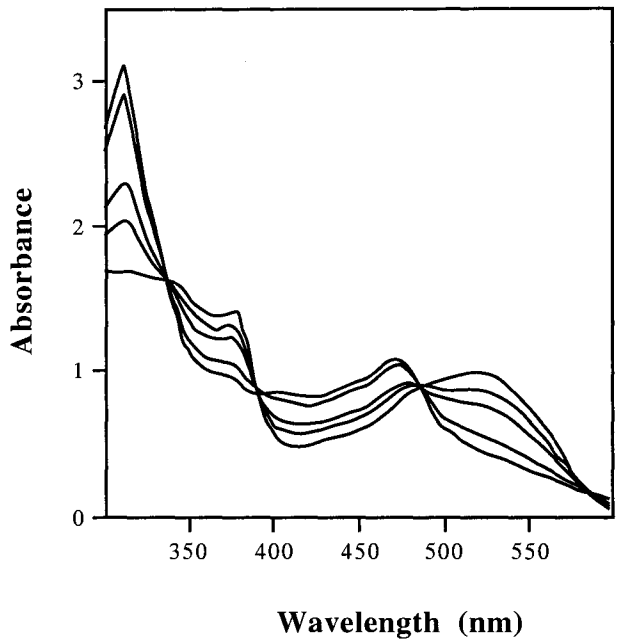


Figure 3.S4 The UV-visible spectra of an 8-MeOAdoCbl thermolysis reaction at 90 °C at 0, 8.00, 16.00, 52.58, and 103.42 hours. Isosbestic points are observed at 338, 392, 486, and 582 nm indicating clean conversion to Co(II)Cbl•.

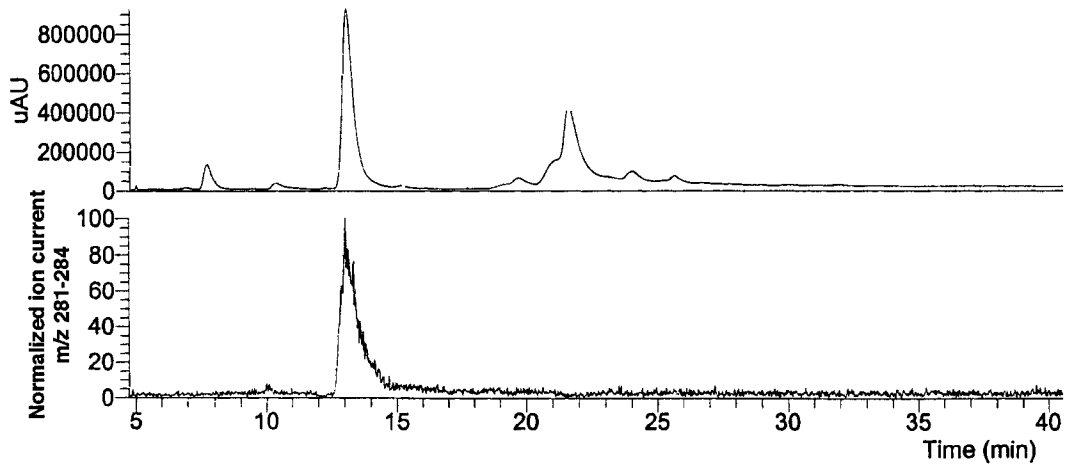


Figure 3.S5 A representative HPLC-MS from a 100 °C thermolysis reaction of 8-MeOAdoCbl in 85% ethylene glycol-d_4. The top signal is a UV-visible absorbance at 254 nm vs time trace. The bottom is the ion current from m/z 281-284 showing the large peak with retention time ~ 13 min contains 8-MeOAdo-H(D). The small peak at ~ 7 min in the UV-visible trace corresponds to a small amount of the expected adenine heterolysis product, and the peak at ~ 22 min corresponds to the products from the Co(II)Cbl•.

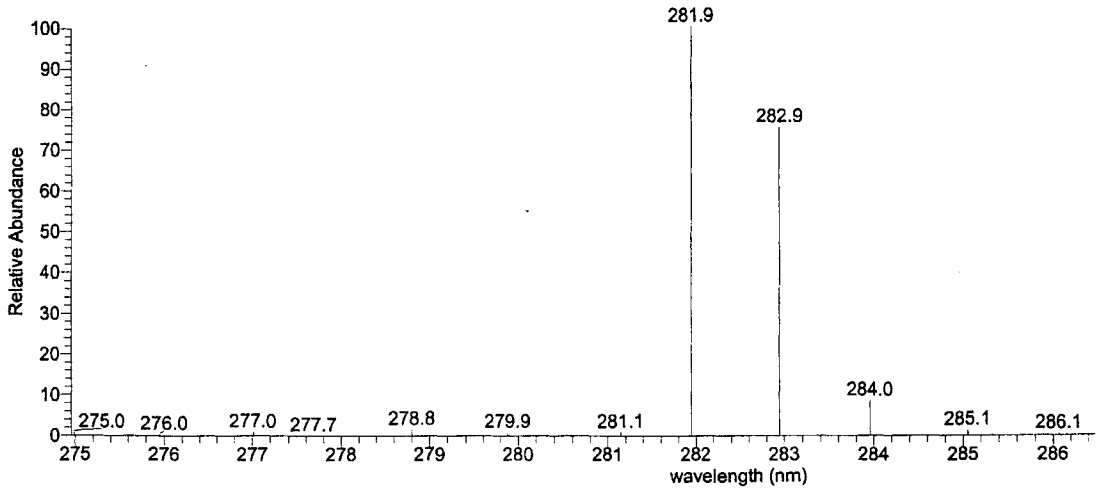


Figure 3.S6 A representative comparison of the ratio of peaks corresponding to 8-MeOAdo-H (281.9) and 8-MeOAdo-D (282.9) from the HPLC-MS (Figure 3.S5) from a 100 °C thermolysis reaction of 8-MeOAdoCbl in 85% ethylene glycol-d_4. The results show, that even in 85% ethylene glycol-d_4, more of the abstraction product is protiated.

120 °C	110 °C	100 °C	90 °C	80 °C
2.64	2.81	3.04	2.60	2.72
2.61	2.74	2.80	2.89	2.76
2.46	2.65	2.81	2.72	2.80
2.52	2.76	2.37	2.71	2.79
2.73	2.82		2.65	2.76
2.71	2.86			2.62
2.75				
2.56				
2.64				
2.95				
2.74				
2.95				
3.00				
Mean	2.71	2.77	2.76	2.71	2.74
Sigma	0.17	0.07	0.27	0.11	0.07

Table 3.S1 The ratio c-Ado/Ado-H in post-thermolysis Ado-B_{12} in ethylene glycol-d_0 over the temperature range 80 °C to 120 °C showing the invariance of this ratio over the 40 °C temperature range studied.

	120 °C	110 °C	100 °C	90 °C	80 °C
	22.2	23.0	25.1	26.6	35.0
	21.8	22.8	24.7	26.9	33.0
				28.9	37.3
				29.9	28.7
				31.1	37.2
				29.5	33.9
				25.9	
				31.1	
Mean	22.0	22.9	24.9	28.7	34.0
Sigma	0.3	0.1	0.3	2.0	3.0

Table 3.S2 The ratio c-Ado/Ado-D in post-thermolysis Ado-B_{12} in ethylene glycol-d_4 over the temperature range 80 °C to 120 °C showing an inverse temperature dependence of this ratio over the 40 °C temperature range studied.

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CHAPTER 4

A Rare Experimental Test of the Hypothesis that Enzymes Have Evolved to Enhance Quantum Mechanical Tunneling in Hydrogen Transfer Reactions: The β-Neopentylcobalamin System

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Intended for submission to *Inorganic Chemistry*

This chapter is intended for submission to *Inorganic Chemistry* with only modifications to the margins and figure numbering for compliance with that journal's requirements. This chapter completes the enzyme-free experiments required to test Klinman's hypothesis: that enzymes may have evolved to enhance quantum-mechanical (QM) tunneling.[1]

Previously, the AdoCbl analog β-neopentylcobalamin (β-NpCbl)[2] was synthesized as its hydrochloride salt (β-NpCbl•H^+Cl^-) and its thermolysis reaction examined.[3] Building off that work, the thermolysis of β-NpCbl in ethylene glycol is studied herein with a focus on measuring the well-established criteria for QM tunneling: a deuterium kinetic isotope effect larger than 6.4 at 20 °C, an activation energy difference greater than 1.2 kcal mol^{-1}, and a ratio of Arrhenius pre-exponential factors less than 0.7.[4,5] The β-NpCbl system has the added advantage that the thermolysis can be studied in the same temperature range where the B_{12}-enzymes operate (5 to 40 °C), allowing a direct comparison of the two sets of data without any extrapolation from higher temperature thermolysis data.

The results allow three important conclusions: the simple neopentyl radical (Np•) displays the same level of tunneling in hydrogen abstraction reactions from ethylene glycol as was seen for the 5'deoxy-adenosyl radical, Ado• (i.e., in data extrapolated to 20 °C from higher temperature thermolyses of AdoCbl; see Chapter Three); there is, therefore, no special enhancement of tunneling by the biological Ado• in comparison to the simple Np• radical; and, overall and most importantly, a comparison of the data for

the three cobalamin solution systems (AdoCbl, 8-MeoAdoCbl, and β-NpCbl) to literature B_{12}-enzyme data reveals that AdoCbl-dependent enzymes are *not* enhancing QM tunneling. Instead, these enzymes are simply exploiting the same level of QM tunneling that is already taking place in Ado$^{\bullet}$-mediated H$^{\bullet}$-atom-abstraction reactions in enzyme-free solution.

Abstract

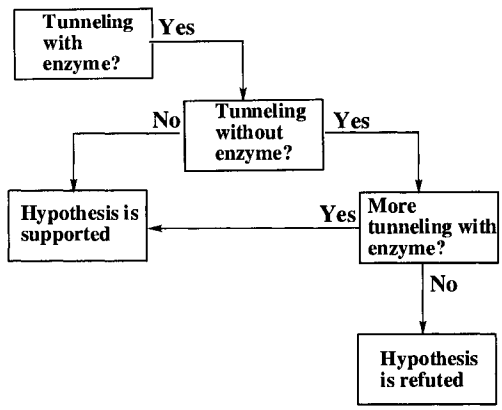
An intriguing but controversial hypothesis has appeared that "The optimization of enzyme catalysis may entail the evolutionary implementation of these chemical strategies that increase the probability of tunneling and thereby accelerate the reaction rate" (Kohen, A.; Klinman, J. P. *Acc. Chem. Res.* **1998**, *31*, 397). Restated, enzymes may have evolved to enhance quantum mechanical tunneling in, for example, their $H^{\bullet}$ atom-abstraction reactions. An alternative hypothesis is that enzymes do not enhance tunneling, but simply exploit the same amount of tunneling present in their enzyme-free solution reactions, if those reactions exist. A third hypothesis is that enzymes actually *decrease* the amount of tunneling as an undesired result of increasing the barrier width while reducing the barrier height. Testing these hypotheses requires the rare event of being able to measure the amount of tunneling in both the enzyme system *and* in a very similar if not identical reaction in enzyme-free solution. This has been accomplished *experimentally* in only one prior case, our recent study of AdoCbl (Coenzyme B_{12}) and 8-Meo-AdoCbl undergoing enzyme-like $H^{\bullet}$ abstraction reactions (Doll, K. M.; Bender, B. R.; Finke, R. G. *J. Am. Chem. Soc.* submitted). The data there reveal *no change* in the leveling of tunneling in or out of the enzyme in comparison to the best literature data for an AdoCbl-dependent enzyme, methylmalonyl-CoA mutase. However, that first system suffers from: the measurement of the KIE (kinetic isotope effect) data in an non-enzymic 80-110 °C temperature range; and lower precision data than desired due to the HPLC or HPLC-MS methods required for the KIE analysis. These limitations have now been overcome by the synthesis, then thermolysis and KIE study vs temperature, of the $H^{\bullet}$ abstraction reaction of β-neopentylcobalamin (β-NpCbl) in ethylene glycol-d_0 and

ethylene glycol-d_4. This is the first experimental test of Klinman's hypothesis using KIE data obtained at enzyme-relevant temperatures. The key data obtained are: deuterium KIEs of 23.1 ± 3.0 at 40 °C to 39.0 ± 2.3 at 10 °C; an activation energy difference E_D-E_H of 3.1 ± 0.3 kcal mol^{-1}; and a pre-exponential factor ratio A_H/A_D of 0.14 ± 0.07. The results are identical within experimental error to the analogous results obtained recently (*op. cit.*) for the AdoCbl and 8-Meo-AdoCbl systems. Significantly, the results are also identical to those for methylmalonyl-CoA mutase, results which yield the definitive finding that there is *no* enzymic enhancement of the tunneling in at least this B_{12}-dependent enzyme. The enzymes do, however, exploit the same (unchanged) level of tunneling measured for the non-enzymic, $Ado^{\bullet}$ solution $H^{\bullet}$ abstraction reaction.

Introduction

There is considerable interest in the study and understanding of quantum mechanical (QM) tunneling in enzymatic hydrogen transfer reactions[1,6-24] including in AdoCbl- (Coenzyme B_{12}-) dependent reactions.[25,26] An intriguing but controversial[6,27-32] hypothesis proposed by Klinman is that "The optimization of enzyme catalysis may entail the evolutionary implementation of these chemical strategies that increase the probability of tunneling and thereby accelerate the reaction rate".[7] Restated, enzymes may have evolved to enhance QM tunneling in, for example, the H•-atom abstraction reactions catalyzed by enzymes such as the AdoCbl-dependent enzymes.[25,33-38] This hypothesis is often cited as reasonable[6,20,24,39-41] since enzymes have many low-frequency motions[42-51] that might well couple to a R•···•H reaction coordinate, making the R•···•H distance smaller, thereby reducing the barrier width and enhancing tunneling.[6,24,40,41]

The criteria often used to identify QM tunneling in hydrogen-transfer reactions were established by Kreevoy[4,5] in 1992. The three Kreevoy criteria diagnostic of tunneling are: (i) a deuterium kinetic isotope effect (k_H/k_D; KIE) significantly larger than the ground-state zero-point energy (GS-ZPE) KIE maximum (6.4 at 20 °C, or 8.9 at 20 °C if secondary isotope effects are included); (ii) an activation energy difference (E_D-E_H) greater than 1.2 kcal mol^{-1}, and (iii) a ratio of pre-exponential factors (A_H/A_D) less than 0.7. The first criterion simply requires the measurement of the KIE while the second two criteria can be obtained from the measurement of the isotope effect as a function of temperature and, then, an Arrhenius plot of ln KIE vs 1 / temperature. The value E_D-E_H is obtained from the slope while A_H/A_D is obtained from the intercept (see section 4.S-1 in the Supporting Information).



Scheme 4.1 A simple three-step procedure for testing the hypothesis[1] that enzymes have evolved to enhance quantum-mechanical tunneling.

The three Kreevoy criteria have been used successfully by Klinman[1,12,19] in eight different enzyme systems. In addition, there is strong experimental evidence for QM tunneling in four AdoCbl-dependent enzymes, diol-dehydratase,[33-36] ethanolamine ammonia lyase,[37,38] glutamate mutase,[52-55] and methylmalonyl-CoA mutase.[25] The most definitive results are in a report by Banerjee and co-workers[25] studying QM tunneling in methylmalonyl CoA mutase (MMCo-A)[25] by Kreevoy's criteria.[4,5] Their key results are a KIE of 35.6 at 20 °C, an activation energy difference of ~3.4 kcal mol^{-1}, and of pre-exponential factor ratios less than 0.08 (row 4 in Table 4.1, vide infra). However, the question remains as to whether or not the B_{12}-dependent or other enzymes have evolved to enhance QM tunneling.

Upon reflection, we and others[12,32,56] realized that a simple yet definitive test of Klinman's intriguing hypothesis (vide supra) can be accomplished *experimentally*[57,26,32] *only* by a comparison of the degree of tunneling in a given enzymic system to the amount of tunneling for that same reaction in the enzyme-free reaction, Scheme 4.1. However, the *problem* in applying the methodology in Scheme 4.1 lies in finding enzymatic

reactions where the identical or nearly identical reaction can be studied *without* the enzyme.[12] Extremely slow rates, or reactions that simply cannot be reproduced without the enzyme present, are among the reasons why the ostensibly simple and definitive test in Scheme 4.1 has not been previously applied *experimentally* save in one recent case.[58] The one exception is our recent experimental test[58] of the Klinman hypothesis using AdoCbl-dependent H$^{\bullet}$ abstraction reactions from ethylene glycol.[59,60-62] Specifically, AdoCbl and, separately, 8-MeOAdoCbl were thermolyzed in a mixture of ethylene glycol-d_0 and deuterated ethylene glycol-d_4 at temperatures from 80-120 °C. This allowed us to measure Kreevoy's three criteria[4,5] diagnostic of QM tunneling using HPLC in the AdoCbl system and using HPLC-MS in the 8-MeOAdoCbl system. The results indicated *no enhancement* of QM tunneling in Ado$^{\bullet}$- mediated H$^{\bullet}$ abstraction reactions in solution in comparison to Banerjee's enzymic-Ado$^{\bullet}$-mediated H$^{\bullet}$ abstraction reactions.[58] However, because our solution reaction has a measurable rate only from 80 to 120 °C, the KIEs measured in our report had to be *extrapolated* to lower temperatures for comparison to the enzymic KIEs measured between 5-40 °C. This is a potential source of error since the ln KIE vs 1 / temperature plots are not truly linear, at least over large temperature ranges, in systems which display tunneling.[4,63] Another problem is that the *HPLC*-MS method required for analysis of KIE data did not provide data as precise as desired, a difficulty which has been reported by others in the literature.[64,65] A third issue unanswered in the Ado$^{\bullet}$ based system is whether or not simple alkyl radicals, R$^{\bullet}$, display a different level of tunneling vs the biological Ado$^{\bullet}$—that is, has Ado$^{\bullet}$ been selected by evolution due to any special tunneling ability in its H$^{\bullet}$-atom abstraction reactions?

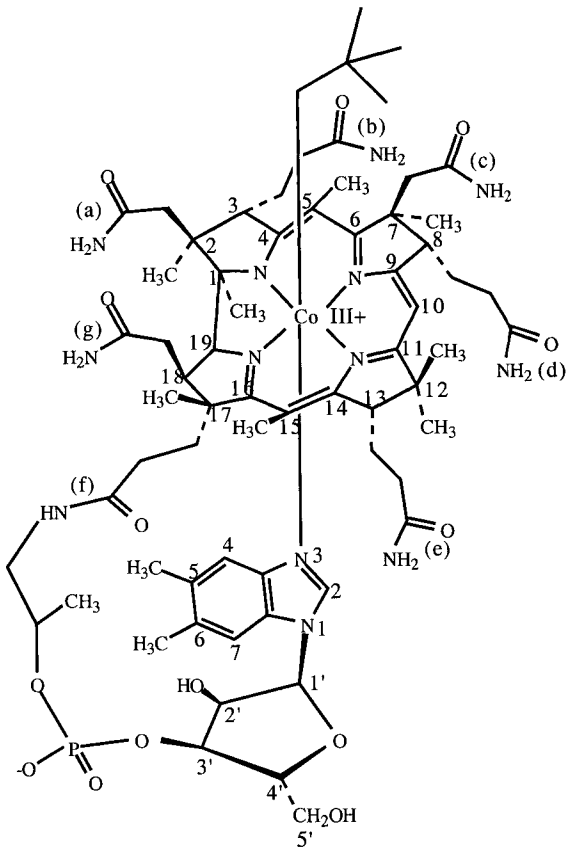


Figure 4.1 The structure of β-Neopentylcobalamin (β-NpCbl) including the IUPAC numbering system of the corrin ring.

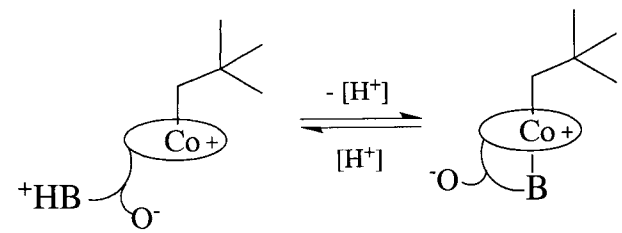
Herein we overcome the primary limitation of the AdoCbl system[58] with a study of β-neopentylcobalamin (β-NpCbl), Figure 4.1. The β-NpCbl system is special in that its thermolysis: (i) has a measurable rate at temperatures from 10-40 °C, thereby allowing us to compare directly KIEs with Banerjee's enzyme values[25] measured in a similar temperature range (5-40 °C); (ii) the neopentane (Np-H[D]) product can be followed by *GC*-MS which should provide data of higher precision (β-NpCbl thermolysis and Co-C bond scission produces Co(II)Cbl$^{\bullet}$ and neopentyl radical (Np$^{\bullet}$), the Np$^{\bullet}$ then abstracts a

H•-atom from the ethylene glycol-d_0 or $-d_4$ substrate producing Np-H(D)). An additional special feature of the β-NpCbl system is that (iii) it allows us to test whether or not a simple alkyl radical, Np•, displays tunneling similar to that observed for the electronically different, more electron deficient Ado• radical.[66-68]

Overall, the β-NpCbl system examined herein is the first experimental test of Klinman's hypothesis with enzyme-free data obtained at enzyme-relevant temperatures. The results are of considerable significance in that Klinman's hypothesis is definitively refuted, at least for the AdoCbl-dependent methylmalonyl-CoA mutase enzyme used in the comparison:[25] QM tunneling of this enzyme's H• abstraction reactions *are not* enhanced. Instead, this enzyme simply exploits the existing, *unchanged* level of QM tunneling that we detect in the analogous enzyme-free, solution H•-atom abstraction reaction.

Results and Discussion

Synthesis of (β-NpCbl•H$^+$Cl$^-$). β-neopentylcobalamin hydrochloride (β-NpCbl•H$^+$Cl$^-$) was synthesized from desalted hydroxocobalamin according to the literature procedure,[3,69-71] with two precautions. Freshly prepared neopentyliodide[2] was synthesized specifically to avoid MeI impurities often present in commercial neopentyliodide.[72],[73,74] The use of neopentylbromide was also avoided due to the its potential to make the α-NpCbl isomer (where the neopentyl group is on the opposite side of the corrin ring).[75-77] The β-NpCbl•H$^+$Cl$^-$ product was isolated in its stable, base-off form, a synthetically useful, isolable form since it undergoes Co–C bond homolysis at least 100 times slower that the deprotonated, base-on form.[78]

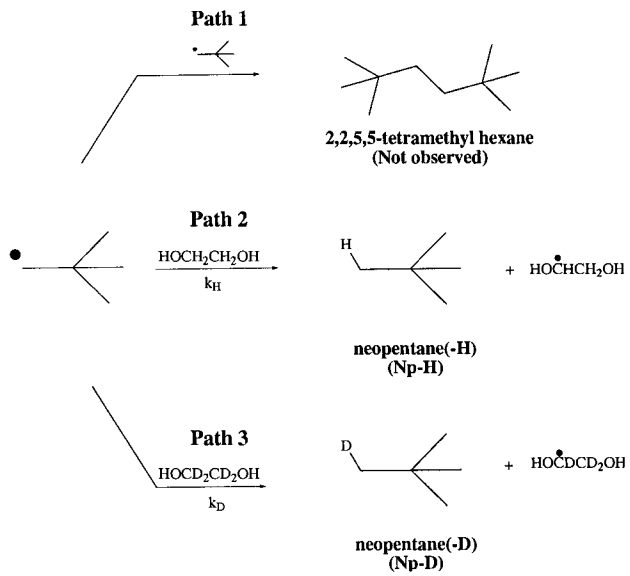


less reactivebase-off form more reactivebase-on form

Scheme 4.2 Reaction scheme for the equilibrium of the less reactive base-off β-NpCbl•H$^+$Cl$^-$ to the more reactive, base-on β-NpCbl which then undergoes homolysis to produce Np$^•$ and Co(II)Cbl$^•$.

Thermolysis of β-NpCbl in Ethylene Glycol-d_0 and $-d_4$. Solutions of β-NpCbl (~10^{-4} M) in a 10% ethylene glycol / 90% ethylene glycol-d_4 buffered with phosphate (0.02 M NaH_2PO_4, 0.03 M Na_2HPO_4, calculated pH 7.4) were thermolyzed at temperatures ranging from 10 to 40 °C in airtight Schlenk cells. The purpose of the

phosphate buffer is to deprotonate the NpCbl•H^+Cl^- (its pKa is between 4.55 and 5.18),[3,71,79,80] thereby shifting the base-off / base-on equilibrium (Scheme 4.2) to the ca. 100-fold more reactive base-on form.



Scheme 4.3 The reactions of Np• in a solution of ethylene glycol and ethylene glycol-d_4.

The Co–C homolysis produces Co(II)Cbl• (monitored by UV-visible spectroscopy) and Np• (monitored indirectly as Np-H(D) by GC-MS, vide infra). The reactions following Co-C homolysis in β-NpCbl proceed as indicated in Scheme 4.3 (the rate laws for the appearance of products and the disappearance of β-NpCbl are given in the Supporting Information, Section 4.S-2). A control experiment in the presence of the radical trap TEMPO gave identical results to those published earlier (Figure 4.S1 in the Supporting Information).[3] There is no detectable Np• dimerization product, Np-Np, from path one (detection limit ~ 10^{-8} M), as the concentration of Np• is never sufficient to

allow this bi-molecular pathway to effectively compete with the hydrogen / deuterium abstraction reactions shown in paths two and three.

Because ionized Np-H and Np-D are not directly detectable[81,82] by GC-MS, we detected the $Me^{\bullet}$ loss fragment peaks at m/z = 57, corresponding to $[(CH_3)_3C]^{\bullet+}$, and at m/z = 58, corresponding to $[(CH_3)_2(CH_2D)C]^{\bullet+}$ (see scheme 4.S1). The KIE was calculated from these two observable m/z = 57 and m/z = 58 peaks using equation 4.1. Note that this equation includes the necessary correction to account for some loss of the deuterium label during the $Me^{\bullet}$ fragmentation (a derivation of this unexceptional equation is available in section 4.S-2 of the Supporting Information.)

$$KIE = \frac{k_H}{k_D} = \frac{\dfrac{100 - (\text{m/z}: 58\ \text{peak}_{\text{corrected}} \times 0.33)}{0.10}}{\dfrac{(\text{m/z}: 58\ \text{peak}_{\text{corrected}} \times 1.33)}{0.90}}$$

Equation 4.1

KIE vs Temperature Results: The Three Kreevoy Criteria. The results yield a large KIE for the hydrogen abstraction of 23 (40 °C) to 39 (10 °C) from the plot of KIE vs temperature, Figure 4.S2 in the Supporting Information. As in the AdoCbl and 8-MeoAdoCbl systems,[58] the KIEs measured in the β-NpCbl system are ~4 fold larger that the predicted maximum GS-ZPE KIEs of 7.9 at 40 °C or 9.5 at 10 °C. (These GS-ZPE maximum KIEs were calculated using a version of the Bigeleisen equation $k_H/k_D \approx \exp\wedge[hc/2k_BT\ (\nu_{CH}-\nu_{CD})]$,[74] they include estimated secondary KIEs of 1.15 and 1.1. A C–H stretching frequency of 2891 cm^{-1} and a C–D stretching frequency of 2137 cm^{-1} were used in the calculation.[83]) A plot of ln KIE vs 1 / temperature was made, Figure 4.2, and an activation energy difference (E_D-E_H) of 3.0 ± 0.3 kcal mol^{-1} and a ratio of pre-

exponential factors (A_H/A_D) of 0.14 ± 0.07 were obtained from the slope and the intercept, respectively.

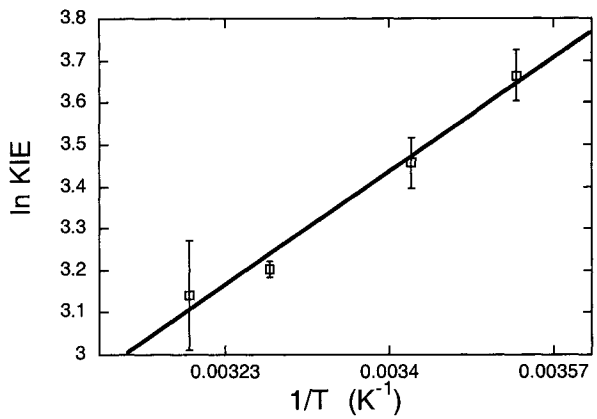


Figure 4.2 A plot of ln KIE vs 1/T for the thermolysis and H•(D•) abstraction reactions of β-NpCbl. From the slope and intercept, the activation energy difference and the pre-exponential factor ratio given in the text were calculated.

The values of the KIE (28 to 39), the activation energy difference ($[E_D-E_H]$ = 3.1 ± 0.3 kcal mol^{-1}), and the pre-exponential factor ratio (A_H/A_D= 0.14 ± 0.07) *all signify tunneling* by Kreevoy's[4] criteria. Moreover, if plots the KIE vs temp of the three, Np•, Ado• and 8-MeOAdo• systems on the same graph, Figure 4.3, it appears that there is no significant difference in the tunneling displayed by these three radicals.

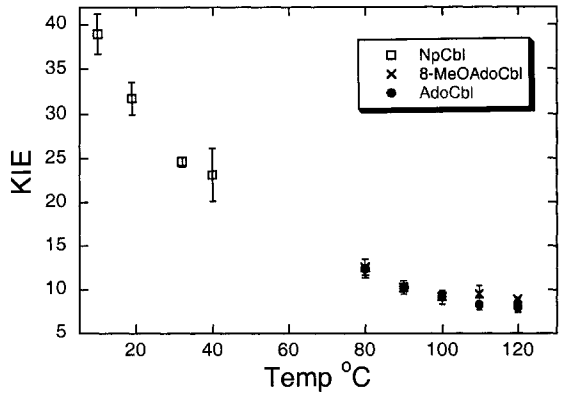


Figure 4.3 A plot of the observed kinetic isotope effects (KIEs) of the hydrogen abstraction from ethylene glycol vs temperature from this work Np• (□), and from previous work[58] Ado• (•), and 8-MeOAdo• (X). The composite data span a temperature range of 120 ^{0} C.

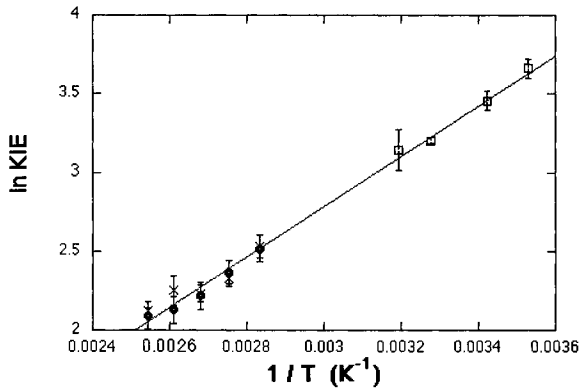


Figure 4.4 A plot of ln KIE vs 1/T using the thermolysis data for all three cobalamin systems, Np• (□), and from previous work[58] Ado• (•), and 8-MeOAdo• (X). From the slope and intercept, the activation energy difference and the pre-exponential factor ratio given in the text were calculated.

A quantitative comparison of KIEs, activation energy differences and pre-exponential factors for the three radicals in Table 4.1 confirms that each does indeed give *identical tunneling within experimental error*. The lack of any detectable difference between the simple Np$^{\bullet}$ and the somewhat electron deficient Ado$^{\bullet}$ is noteworthy.

Plotting the ln KIE vs 1/T for all three cobalamins (Figure 4.4) gives a plot that appears to be linear over a temperature range of 120 °C. An activation energy difference of (3.15 ± 0.08 kcal mol^{-1}) and a pre-exponential factor ratio of (A_H/A_D) = 0.13 ± 0.02 were calculated using the slope and intercept of this plot. To our knowledge, there is no other data set in the literature with such tunneling data measured over such a large temperature range.

As a control, the use of ethylene glycol-d_6 instead of ethylene glycol-d_4 was also performed with no significant changes in the results (KIE = 21.8 at 40 °C). This experiment shows that the hydrogen abstracted is, as anticipated, from a C–H(D) bond of ethylene glycol and not from its O-H(D) bond.[84,85] It also rules out the hypothesis that the Np$^{\bullet}$ is abstracting a hydrogen from the exchangeable hydrogens in the phosphate buffer.

Table 4.1 The observed KIE, activation energy difference, and pre-exponential factor ratio for the β-NpCbl system, for the previous enzyme-free systems (AdoCbl and 8-MeOAdoCbl), and for literature AdoCbl-dependent enzyme systems.

	KIE	E_D-E_H (kcal mol^{-1})	A_H/A_D
NpCbl in solution	35.2 ± 1.8 at 19 °C 39.0 ± 2.3 at 10 °C	3.1 ± 0.3	0.14 ± 0.07
AdoCbl in solution[58]	12.4 ± 1.1 at 80 °C ~29.3 at 20 °C [a] ~35.2 at 10 °C [a]	3.0 ± 0.3	0.16 ± 0.07
8-MeOAdoCbl in solution[58]	12.5 ± 0.9 at 80 °C ~21.8 at 20 °C [a] ~24.8 at 10 °C [a]	2.1 ± 0.6 [b]	0.5 ± 0.4 [b]
Composite of all three cobalamins	29 ± 5 at 20 °C [c] 38 ± 7 at 10 °C [c]	3.15 ± 0.07	0.13 ± 0.02
Methylmalonyl-CoA mutase[25]	35.6 at 20 °C ~12.7 at 80 °C [d]	3.41±0.07 (3.54 ± 0.19) [e]	0.078±0.009 (0.082 ± 0.28) [e]
Ethanolamine ammonia lyase[37,38]	> 10	(3.1 ± 1.1) [f]	(0.038 ± 2.13) [f]
Glutamate mutase[52,55]	28 to 35 at 10 °C	NA [g]	NA [g]
diol dehydratase[34,56,86,87]	8 and 28.6 at 10 °C	NA [g]	NA [g]
GS-ZPE 1° only	6.4 at 20 °C 6.8 at 10 °C	1.2	1.0
GS-ZPE 1° and 2°	8.9 at 20 °C [h] 9.5 at 20 °C [h]	1.2	1.0

[a] Values are extrapolated from the higher temperature data and represent lower limits to the true values.
[b] The larger error bars for this 8-MeOAdoCbl data set are due to the intrinsically larger errors of the ion-trap HPLC-MS method used to analyze the products.[64,65]
[c] Calculated from the value and error of the linear regression in Figure 4.4.
[d] This 80 °C value was obtained by extrapolation from the lower temperature data set.
[e] These are our linear-regression analysis of the literature data set.[25] See the Supporting Information elsewhere[58] for further information.
[f] These numbers were calculated from the data in the literature.[38] See the Supporting Information elsewhere[58] for further information.
[g] NA = not available in the literature.
[h] The GS-ZPE 1^{o} and 2^{o} KIE = $[(1^{o}\text{KIE}) \times 1.15 \times 1.1^{2}]$.

Conclusions

Herein we have shown: (i) that β-NpCbl is a ideal, low-temperature Co-C thermolysis system (10-40 °C) which allows the acquisition, for the first time, of data in the same temperature range where the KIEs for the B_{12}-dependent methylmalonyl-CoA mutase enzyme system has been measured, (ii) that the resultant GC-MS KIE vs temperature data is of as high a precision as we have been able to obtain previously by HPLC-MS for at least the 8-MeO-AdoCbl system;[58] and (iii) that each of the three H• abstraction systems, Np•, Ado• and 8-MeO-Ado•, yield identical results within experimental error, thereby showing that there is nothing special about—and, specifically there is no enhancement of tunneling by—Ado• over the simple alkyl radical, Np•. Hence, there is no evidence for any evolutionary selection of Ado• due to some special tunneling prowess. Moreover, (iv) we have k_H/k_D isotope effect data over a *120 °C temperature range* for our model study, data which allows the calculation of rather precise E_D-E_H = 3.15 ± 0.08 kcal mol^{-1} and A_H/A_D = 0.13 ± 0.02. Such values are now being examined to see if we can extract relatively rare *experimental* information about the barrier height and width of this reaction. Finally and most importantly, our results show: (v) that within experimental error (Table 4.1), a direct comparison of the enzyme-free and RCbl (R = Ado, 8-MeOAdo, and Np) systems to the AdoCbl-dependent enzymic data of Banerjee and co-workers *reveals no statistically significant increase in any of the three Kreevoy criteria for tunneling.* This study—the first experimental test of Klinman's hypothesis at enzyme relevant temperatures for very similar reactions[59] inside and outside of the enzyme—makes it clear that at least B_{12}-dependent methylmalonyl-CoA mutase *has <u>not</u> evolved to enhance QM tunneling in its H• abstraction reactions.*

The enzyme does, however, exploit within experimental error the exact same level of QM tunneling available in the non-enzymatic solution reaction.

It remains of interest to try to obtain the Kreevoy criteria for the enzyme B_{12}-dependent diol dehydratase so that an exact comparison of enzymic data for the identical reaction[59] ($H^{\bullet}$ abstraction from ethylene glycol) can be made. It also remains to be seen if similar conclusions can be obtained for other enzymic systems and by the methodology detailed in Scheme 4.1.[88]

Experimental

Materials

The following were used as received: neopentyl alcohol (Aldrich, 99%), sodium iodide (Aldrich, 99.999%), calcium hydride (Aldrich 90-95%), phenol (Acros, ACS grade), benzene (Aldrich, HPLC grade), hexane (Fisher Scientific, ACS grade), chloroform (Fisher Scientific, ACS grade), hydrochloric acid (Mallinckrodt, AR grade), ethyl ether (Fisher Scientific, ACS grade, anhydrous), pyridine (Aldrich, 99.8%, anhydrous), tosyl chloride (Aldrich, 99%), TEMPO (Aldrich 99% sublimed), methanol (Fisher Scientific, HPLC grade), argon (General Air), ethylene glycol-d_6 (Cambridge Isotope Labs, 98%), and 2,2,5,5-tetramethyl hexane (Chemsampco). Diglyme (Aldrich, 99.5%, anhydrous) was stirred over CaH_2 for 12 hours then distilled under argon. The purities of ethylene glycol-d_0 (Aldrich, 99.8% anhydrous) and ethylene glycol-d_4 (Cambridge Isotope Labs, 98%) were confirmed by GC-MS; hence, these were used as received. Distilled water was filtered through a Barnstead nano-pure filtration system.

Instrumentation and Equipment

UV-visible absorption spectra (± 1 nm) were recorded on a Hewlett-Packard Model 8452A UV-visible diode array spectrophotometer equipped with a thermoelectric Hewlett-Packard 89090A Peltier cell block temperature controller operating at 25.0 ± 0.1 °C. GC-MS was performed on an Agilent 5973N/6890 with a 30 m Agilent HP-5 column. All linear regressions were performed on a Power Macintosh 5400/120 using Microsoft Excel 98.

Thermolyses of β-NpCbl•H^+Cl^- were carried out in Schlenk cuvettes[89] prepared by glass blowing PTFE needle valves onto 1 cm path length cuvettes or to 1 mL glass

vials. The cuvettes' ability to maintain an oxygen-free environment was tested with air sensitive Co(II)Cbl• (made from the photolysis of AdoCbl in ethylene glycol). No detectable Co(II)Cbl• decomposition was observed for any of the Schlenk cuvettes employed in this work over the time scale used for our thermolyses (~1 week).

Thermolysis temperatures were maintained by immersing the cuvettes in a VWR model 1166 thermostatted water bath. The temperature of each reaction was verified (± 0.2 ^{o}C) using a mercury thermometer with gradations in the temperature range of interest.

All samples were prepared in a Vacuum Atmospheres inert atmosphere drybox. An O_2 level of < 2 ppm was monitored by a Vacuum Atmospheres model AO 316-C oxygen analyzer.

Alkylcobalamins are very 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.

β-NpCbl•H^+Cl^- Synthesis

β-Neopentylcobalamin was synthesized as its HCl salt, β-NpCbl•H^+Cl^-, by the literature method using freshly prepared neopentyl iodide,[2,3,69,70] yield 37% (literature yield 62% before drying). The product was characterized by: UV-visible spectroscopy in buffered ethylene glycol, [λ-max (ε x 10^{-3} M cm) 388 nm (9500) 438 nm (8000) 486 nm (6900) within experimental error of literature values[79]]; by LSIMS [found m/z = 1400.6 (calculated m/z = 1400.7 for [NpCbl–H^+])]; and by ^{1}H NMR [δ 8.91 (s, 1H-B2), δ 7.49 (s, 1H-B7), δ7.38 (s, 1H-B4), δ6.97 (s, 1H-C-10), δ 6.55 (d, 1H-R1'), in unbuffered D_2O referenced to TSP-d_4], product purity ~ 90%, results all within experimental error to literature values.[79]

Neopentyl iodide (NpI) was prepared by refluxing neopentyl tosylate (NpOTs) with excess NaI in diglyme.[2] The reaction was followed by ^{1}H NMR until its completion, 155 min. Purification was accomplished by vacuum distillation yielding a 20% NpI by ^{1}H NMR in diglyme solution with a purity of the > 95%. This synthetic route was chosen specifically to avoid the use of MeI, a common impurity in commercial NpI, which if present, results in the formation of a methylcobalamin (MeCbl) contamination in the final product, β-NpCbl•H^+Cl^-.[90] It is also important to use NpI rather than NpBr to avoid the possible formation of the unwanted α-isomer, α-NpCbl•H^+Cl^-.[75-77]

Neopentyl tosylate (NpOTs) was synthesized by literature methods[91,92] from neopentylalcohol and tosyl chloride in pyridine. It was recrystallized from a 17:1 mixture of hexanes/benzene at -15°C, yield 81% (literature yield 87%).[91] Purity >95% by ^{1}H NMR.

β-Neopentylcobalamin Thermolysis and Analysis Procedure

The β-neopentylcobalamin thermolysis procedure is similar to our literature thermolysis procedure.[3] β-Neopentylcobalamin hydrochloride (~1.0 mg) was weighed out inside a foil-wrapped vial and brought into the drybox. It was dissolved in ethylene glycol buffer solution (0.02 M NaH_2PO_4 0.03 M Na_2HPO_4, calculated pH 7.4) making a ~2 x 10^{-4} M β-NpCbl•H^+Cl^- solution which was transferred into foil-wrapped, airtight Schlenk cells.[89] The cells that were used in KIE calculations contained 90% ethylene glycol-d_4. The sealed cells were taken into darkroom and thermolyzed in a temperature controlled water bath (10.0, 19.0, 32.0, 40.0 °C) for at least 5 half lives (142, 46, 8, and 2 hrs). One UV-visible Schlenk cuvette was monitored at each temperature; resultant

thermolysis rates were within error of those reported previously.[3] Control experiments were also performed using ethylene glycol-d_6 at 20°C yielding a KIE of 32.8 and at 40 °C yielding a KIE of 20.8. Both of these values are within experimental error of the values measured in the ethylene glycol-d_4 system.

Gas headspace samples (10 uL) were taken from the Schlenk cells and injected into the GC-MS: injector temperature 200 °C; source temperature 160 °C; temperature program: 0 °C for 2.5 min; ramp to 25 °C at 5°C / min; then ramp to 175 °C at 20 °C / min and hold for 1 min until returning to 0 °C; electron energy 70 EV; mass detector set to read m/z = 35-350.

In an important control experiment, solutions of 2,2,5,5 tetramethylhexane in ethylene glycol were prepared, and a detection limit of 10^{-8} M was established, showing that if even 1% of the Np$^{\bullet}$ dimerized (path one in Scheme 4.2), it would have been detected.

Supporting Information

Supporting Information [available from the ACS; it will also be published it in the Ph. D. dissertation of K. M. Doll (Colorado State University Spring 2003)]: Figure 4.S1 The UV-visible spectra of a β-NpCbl thermolysis reaction containing excess TEMPO; Figure 4.S2: A plot of KIE vs temperature for the Np$^{\bullet}$ hydrogen-abstraction reactions; Figure 4.S3: A GC-MS trace of a sample from a NpCbl thermolysis reaction; Section 4.S-1: A short derivation of showing the use of a ln KIE vs 1/T plot to diagnose tunneling; Section 4.S-2 The kinetic rate laws and an expression for the derivation of as expression for the KIE; Section 4.S-3: Derivation of Equation 4.1; Scheme 4.S1: The reaction scheme for the homolysis and subsequent hydrogen abstractions in ethylene glycol / ethylene glycol-d_4; Scheme 4.S2 The fragmentation reactions of Np-H and Np-D in the mass spectrometer leading to the observed products:

Acknowledgments

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atom abstractions comes from the fact that all three enzyme-free systems studied, NpCbl, AdoCbl and 8-MeOCbl, give results that are the same within experimental error to each other as well as to the data for AdoCbl dependent methynlalonyl-CoA mutase.
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Supporting Information

A Rare Experimental Test of the Hypothesis that Enzymes Have Evolved to Enhance Quantum Mechanical Tunneling in Hydrogen Transfer Reactions: The β-Neopentylcobalamin System

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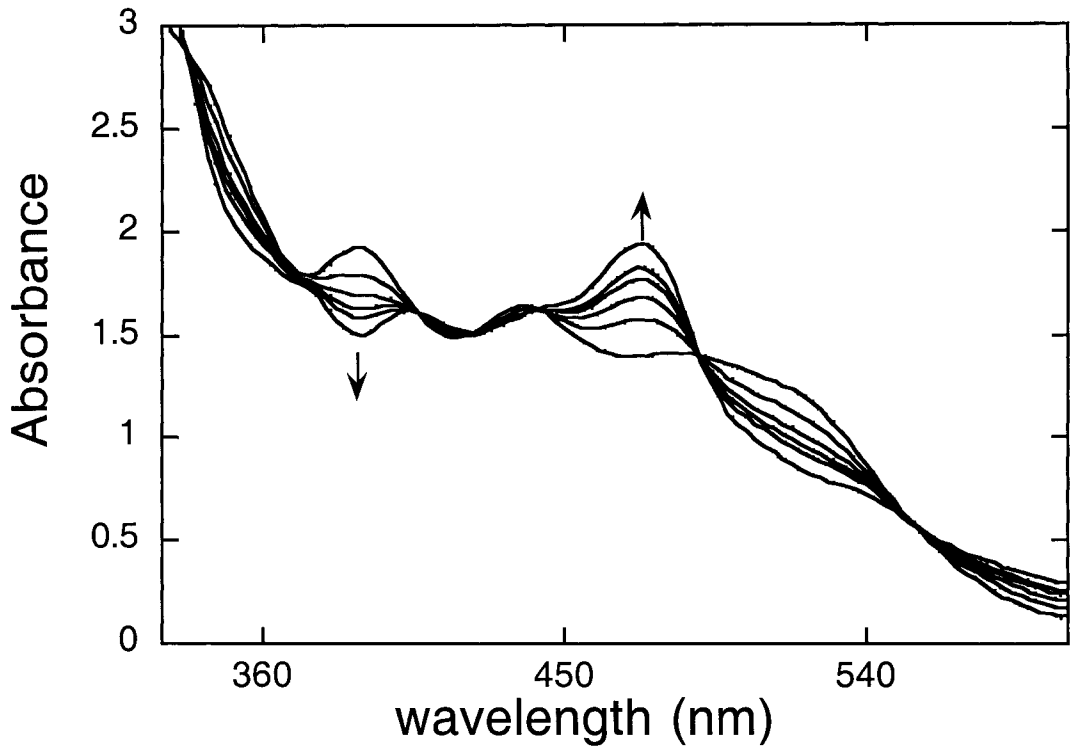


Figure 4.S1. A 25.7 °C thermolysis of β-NpCbl (~2 x 10^{-4} M) in buffered ethylene glycol solution (0.02 M NaH_2PO_4, 0.03 M Na_2HPO_4, calculated pH 7.4) which also contained 2 x 10^{-3} M TEMPO as a radical trap. The spectra shown were taken at t = 0, 1, 2, 3, 4, and 10 hrs. As expected, there are six isosbestic points at 338, 408, 420, 444, 490, and 556 nm showing clean conversion of NpCbl to Co(II)Cbl•. The first-order rate constant calculated for both the disappearance of the peak at 388 nm and the appearance of the peak at 474 nm is 1.0 x 10^{-4} s^{-1}, a value within experimental error (± 0.1 x 10^{-4} s^{-1}) to that obtained in earlier work.[1]

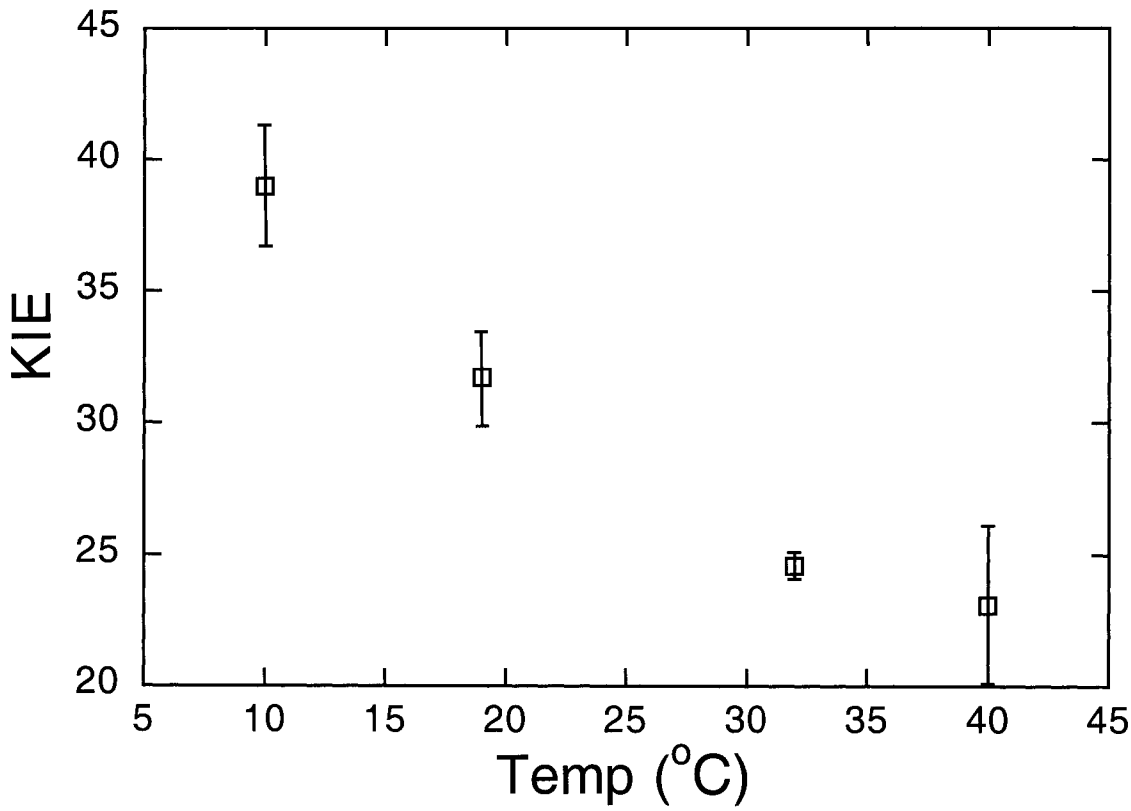


Figure 4.S2. A plot of the KIE vs temperature for hydrogen abstraction from ethylene glycol by Np$^{\bullet}$ generated from the thermolysis of NpCbl. The data reveal the expected higher KIEs at lower temperatures, 39.0 ± 2.3 (at 10 °C) and 23.1± 3.0 (at 40 °C).

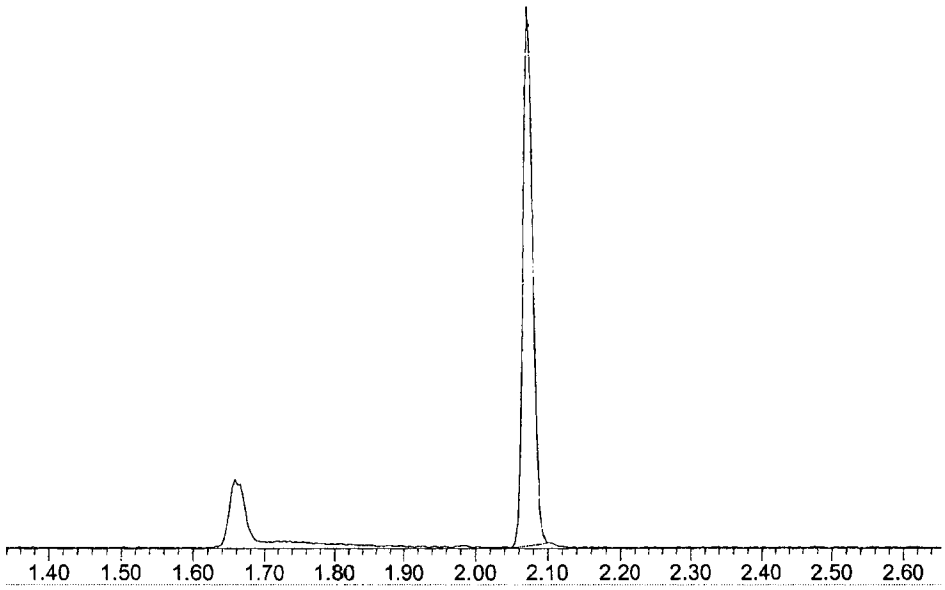


Figure 4.S3. A GC-MS trace of total ion current vs time of the gas headspace sample taken from a 30 °C NpCbl thermolysis in a Schlenk cuvette (see the Experimental section in the main text for details). The peak at ~ 1.66 min is air and the peak at ~ 2.07 min is neopentane. The peak for authentic 2,2,5,5-tetramethylhexane, the Np• dimerization product, has a retention time of ~10 min under identical conditions, but was not detected in any of the reaction solutions, as expected (detection limit ~ 10^{-8} M).

Section 4.S-1. The use of a ln KIE vs 1/T Arrhenius plot to obtain the activation energy difference (E_H-E_D) and pre-exponential factor ratio (A_H/A_D).

The standard definition of a deuterium kinetic isotope effect

$$(1)\quad KIE = \frac{k_H}{k_D}$$

Standard Arrhenius expressions for the rate of k_H and k_D

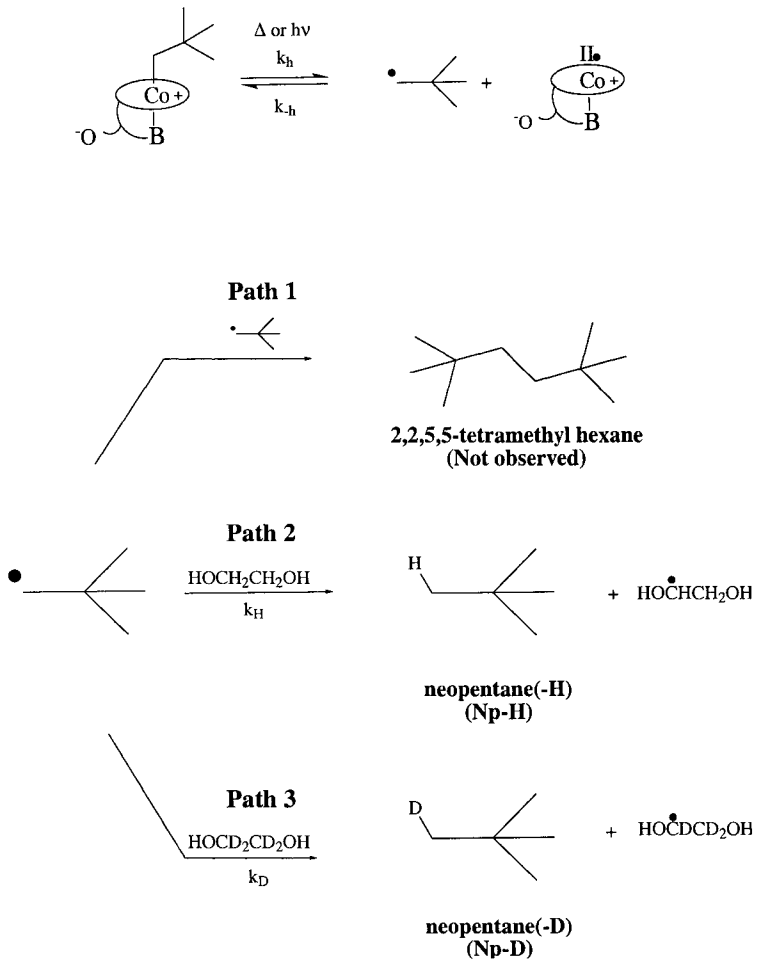
$$(2)\quad k_H = A_H \times e^{\left[\frac{-E_H}{RT}\right]} \qquad (3)\quad k_D = A_D \times e^{\left[\frac{-E_D}{RT}\right]}$$

Substitution of (2) and (3) into (1) gives (4)

$$(4)\quad \frac{k_H}{k_D} = \frac{A_H \times e^{\left[\frac{-E_H}{RT}\right]}}{A_D \times e^{\left[\frac{-E_D}{RT}\right]}}$$

Taking the ln of both sides gives (5). Hence, plot of ln KIE vs 1/T has a slope (E_D-E_H)/R and an intercept of ln (A_H/A_D).

$$(5)\quad \ln(KIE) = \ln\left(\frac{k_H}{k_D}\right) = \ln\left(\frac{A_H}{A_D}\right) + \left(\frac{1}{T}\right)\left(\frac{E_D - E_H}{R}\right)$$



Scheme 4.S.1 The reaction scheme for the homolysis and subsequent hydrogen abstractions in ethylene glycol / ethylene glycol d-$_4$ solution.

Section 4.S-2 The kinetic rate laws and the derivation of an expression for the KIE

Using scheme 4.S.1, and the steady state approximation for Np•, the rate law for the production of Np-H, Np-D, and the disappearance of NpCbl can be obtained; the results are expressions similar to the rate law for the analogous reactions of AdoCbl.[2,3]

$$\frac{-d(NpCbl)}{dt} = k_h[NpCbl]\left[\frac{k_H[EG-d_0]+k_D[EG-d_4]}{k_{-h}[Co(II)Cbl] + k_H[EG-d_0]+k_D[EG-d_4]}\right]$$

$$\frac{d(Np-H)}{dt} = k_h[NpCbl]\left[\frac{k_H[EG-d_0]}{k_{-h}[Co(II)Cbl] + k_H[EG-d_0]+k_D[EG-d_4]}\right]$$

$$\frac{d(Np-D)}{dt} = k_h[NpCbl]\left[\frac{k_D[EG-d_4]}{k_{-h}[Co(II)Cbl] + k_H[EG-d_0]+k_D[EG-d_4]}\right]$$

Derivation of an expression for the KIE for the reactions in Scheme 4.S.2.

The rates of formation of Np-H and Np-D are as follows (ethylene glycol is abbreviated EG):

$$d[\text{Ado-H}]/dt = k_H[Np^\bullet]\,[EG\text{-}d_0] \quad (S1)$$

$$d[\text{Ado-D}]/dt = k_D\,[Np^\bullet][EG\text{-}d_4] \quad (S2)$$

Integrating both sides of S1 and S2,

$$[\text{Np-H}] = k_H\,[EG\text{-}d_0]\int [Np^\bullet]\,dt \quad (S3)$$

$$[\text{Np-D}] = k_D\,[EG\text{-}d_4]\int [Np^\bullet]\,dt \quad (S4)$$

Dividing S3 by S4,

$$[\text{Np-H}]/[\text{Np-D}] = k_H\,[EG\text{-}d_0]/k_D\,[EG\text{-}d4] \quad (S5)$$

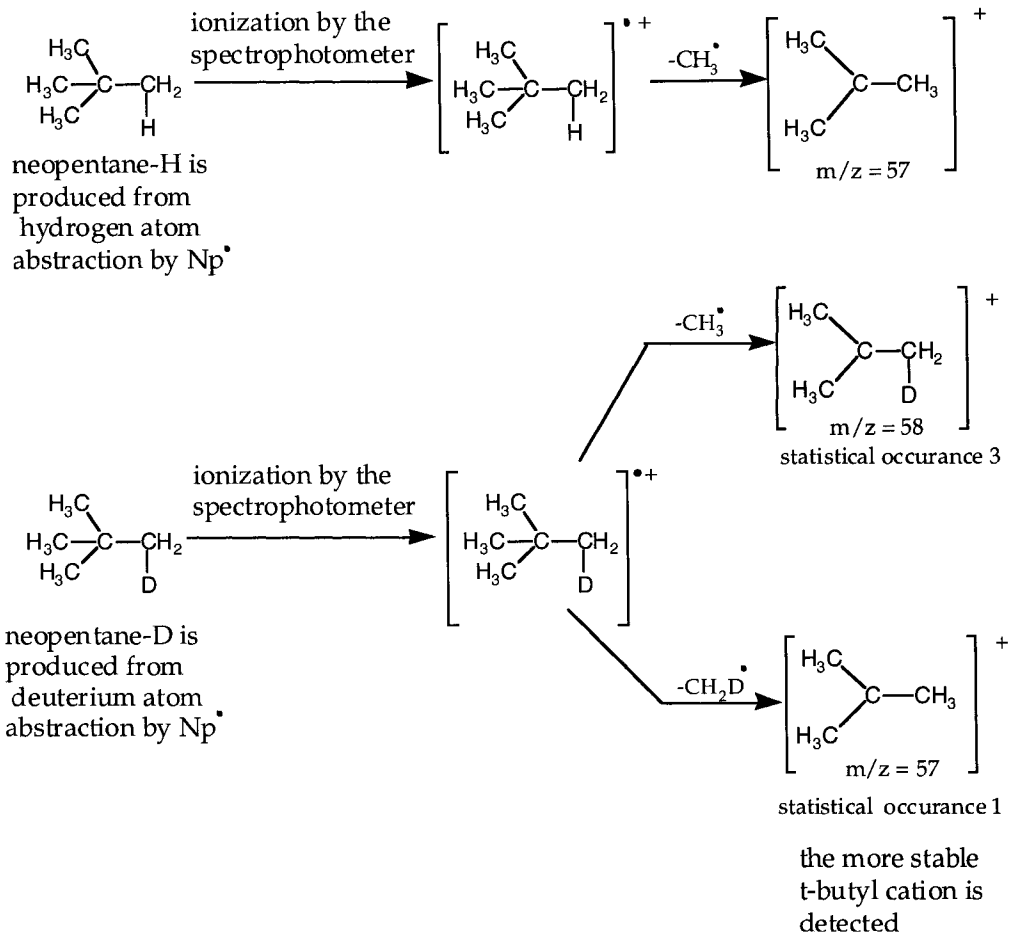
Rearranging S5 gives: $KIE = \frac{k_H}{k_D} = \frac{\frac{[Np - H]}{[EG - d_0]}}{\frac{[Np - D]}{[EG - d_4]}}$

This expression is the starting point for the derivation of Equation 4.1 in Section 4.S-3.

Section 4.S-3 Derivation of Equation 4.1

Ionized neopentane-H and neopentane-D (m/z = 72.1 and 73.1, respectively) are not observed directly in the GC-MS. Instead, the more stable $[(CH_3)_3C]^{\bullet+}$ and $[(CH_3)_2(CH_2D)C]^{\bullet+}$ (m/z = 57 and 58) are detected. In order to quantitate the amount of Np-H and Np-D produced by the H$^{\bullet}$ abstraction reaction, it is necessary to use Equation 4.1, which account the 1-in-4 statistical chance that the deuterium is lost in the fragmentation. In order to use equation 4.1, the following conditions have to apply:

1. The thermolysis reaction solution must have a composition of 10% ethylene glycol, 90% ethylene glycol-d_4. Additionally, the ratio of ethylene glycol / ethylene glycol-d_4 concentration must not change during the reaction. This assumption should cause, at most, a 0.057 % error.
2. The value of the m/z : 57 peak is assigned a relative value of 100.
3. That the $[Np\text{-}D]^+$ decomposes to the m/z : 57 and m/z : 58 peaks in the statistical ratio of 3 to 1, as shown in Scheme 4.S2. That is, we assume that there is no significant secondary isotope effect on the cation decomposition. This assumption should be good to ± 3 %, for a β-secondary KIE close to the value of 1.1.[4,5]



Scheme 4.S2 The full reactions of neopentane-H and neopentane-D in the mass spectrometer which lead to the observed m/z : 57 and m/z : 58 peaks.

The product Np-H must be divided by the percentage of the reaction solution which had an available hydrogen for abstraction (10 %) and Np-D must be divided by the percentage of the reaction solution that had an available deuterium for abstraction (90 %).

$$(1)\quad KIE = \frac{k_H}{k_D} = \frac{\dfrac{\text{Np - H}}{0.10}}{\dfrac{\text{Np - D}}{0.90}}$$

A term of 4.6 must be subtracted from the observed m/z : 58 peak value, to compensate for isotopic contributions to the m/z : 58 peak from the heavier isotopes (i.e., ^{13}C) in the non-deuterated ion.

(2) $\text{m/z : 58 peak}_{\text{corrected}} = \text{m/z : 58peak} - 4.6$

Contribution from the deuterated ion, which lost its deuterium in the fragmentation, must be subtracted from the m/z : 57 peak.

(3) $\text{Np - H} = 100 - (\text{m/z : 58 peak}_{\text{corrected}} \times 0.33)$

The m/z :58 peak must be corrected for deuterium lost in the fragmentation:

(4) $\text{Np - D} = (\text{m/z : 58 peak}_{\text{corrected}} \times 1.33)$

Substitution of (3) and (4) into (1) gives (5)

$$(5)\quad KIE = \frac{k_H}{k_D} = \frac{\dfrac{100 - (\text{m/z : 58 peak}_{\text{corrected}} \times 0.33)}{0.10}}{\dfrac{(\text{m/z : 58 peak}_{\text{corrected}} \times 1.33)}{0.90}}$$

Substitutions equation (2) into (5) gives equation 4.1 in the main text.

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CHAPTER 5

SUMMARY

The first step in any scientific endeavor is to gain sufficient knowledge to allow one to ask an important question. AdoCbl and QM tunneling in hydrogen transfer reactions both have rich histories with many books and hundreds of scientific papers authored on each of these topics. It was the purpose of Chapter One of this dissertation to give a small glimpse of this history by briefly describing some of the literature in AdoCbl-dependent enzymes and QM tunneling which form the foundation for this dissertation.

Next, one must propose a hypothesis which answers the question and, then, think of possible alternative hypotheses.[1] Klinman's hypothesis: "The optimization of enzyme catalysis may entail the evolutionary implementation of these chemical strategies that increase the probability of tunneling and thereby accelerate the reaction rate."[2] proposes an answer to the question: why is QM tunneling observed in many enzymic hydrogen transfer reactions? An alternative hypothesis is that QM tunneling already occurs in this reaction, and the enzyme is not increasing or decreasing that tunneling.

Third, hypothesis-driven science must focus on the careful design and execution of experiments that will rule out alternatives to uncover the correct answer to the question.[1] In chemistry, an early step is often synthesis. Chapter Two describes the synthesis of a molecule 8-MeOAdoCbl which has proven crucial in the experiments described in Chapter Three. Additionally, NpCbl was synthesized and utilized in Chapter Four. Chapters Three and Four contain the experiments designed to test Klinman's hypothesis as well as the main alternative hypothesis noted above. Testing these hypotheses *experimentally* is something which we, and only we, have successfully done.

The last, and equally important, part of science is communication. That is the purpose of this chapter, as well as the dissertation as a whole. I have attempted to condense both the purpose and the important message of this dissertation to these few, summary pages.

This dissertation has accomplished its goal of comparing an enzyme system to the same reaction in an enzyme-free system. Using this data, we can now fill in the blanks on Table 1.4, now shown in Table 5.1.

Table 5.1

A now complete data table[3-12] giving the necessary information for testing Klinman's hypothesis.

	KIE	**E_D-E_H (kcal mol^{-1})**	**A_H/A_D**
Comparable Solution Reactions	**~22-35 at 20 °C ~25-39 at 10 °C**	**2.1-3.1**	**0.14-0.5**
methylmalonyl-CoA mutase	35.6 at 20 °C 43.1 at 10 °C	3.41±0.07	0.078±0.009
ethanolamine ammonia lyase	NA	3.1 ± 1.1	0.038 ± 2.13
glutamate mutase	28 to 35 at 10 °C	NA	NA
diol dehydratase	8 and 28.6 at 10 °C	NA	NA
GS-ZPE	6.4 at 20 °C 6.8 at 10 °C	1.2	1.0

Utilizing our simple, definitive procedure (Figure 1.17) for testing Klinman's hypothesis leads to the conclusion that there is *not* a significant enhancement of QM tunneling of H$^{\bullet}$ abstraction in AdoCbl-dependant enzyme systems. The AdoCbl enzyme does, however, exploit the same level of tunneling available in the enzyme-free system.

The search for other enzyme systems and their enzyme-free reactions where the procedure in Figure 1.17 can be applied is of interest. Such studies will be necessary to test the generality of our "no *enhancement* of tunneling" conclusion. It seems probable, however, that Klinman's controversial and much publicized hypothesis will be refuted. The present dissertation is the first, definitive piece of experimental data in this direction.

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APPENDIX A

Independent Research Proposal

Kenneth M. Doll

Submitted April 12, 2002

Efficiently Labeling DNA by Combining Cyclic Peptide Copper (II) Chelating Ability and DNA Site Selective Binding Ability on a Single Peptide Strand.

Abstract/Specific Aims

Using a peptide that has both a metal chelating domain ((χ-cyclo)-Lys-Ser-Gly-Thz-Ile-Thr-Gly-Thz) and a site specific DNA binding domain, CNC4-br1 [a peptide with the amino acid sequence: (Pro-Glu-Ser-Ser-Asp-Pro-Ala-Ala-Leu-Lys-Arg-Ala-Arg-Asn-Thr-Glu-Ala-Ala-Arg-Arg-Ser-Arg-Ala-Arg-Lys-Leu-Gln-Arg-Met-Lys-Gln-Gly-Gly-Cys)] will combine the advantages of high binding site specificity and versatile ion chelating ability. The use of a single peptide strand, followed by cyclization of the copper (II) chelating domain will allow for easy synthesis. Changes in the DNA binding domain, the metal chelating domain, or both, can be accomplished during the initial peptide synthesis. This should allow the same general method to work for labeling different DNA sequences, or for changing the ion used in labeling.

The overall goal of this research is to develop a method that will help identify a simple peptide that can bind a unique site on a chromosome.

Specific hypotheses:

1. The linear peptide (CNC4-br1- Gly-(χ-cyclo) Lys-Ser-Gly-Thz-Ile-Thr-Gly-Thz) (Figure AA.1) can easily be synthesized.
2. The cyclization of the domain (χ-cyclo)-Lys-Ser-Gly-Thz-Ile-Thr-Gly-Thz) (Figure AA.1) is possible, and it will form two copper (II) binding sites.
3. The DNA binding domain (CNC4-br1) (Figure AA.1) will retain its DNA binding ability and effectively attach two copper (II) ions to a specific DNA binding site.

Specific aims:

1. We will synthesize the peptide (CNC4-br1-Gly-Lys-Ser-Gly-Thz-Ile-Thr-Gly-Thz) by synthesis of the linear peptide, followed by a cyclization reaction. The cyclization will be carried out both with and without $Cu(NO_3)_2$ present to test for a possible templating effect. If necessary, the "safety catch linker"[1,2] reaction for cyclization might also be tried.

2. We will show that (CNC4-br1-Gly-(χcyclo)-Lys-Ser-Gly-Thz-Ile-Thr-Gly-Thz) will bind to a pre-selected small strand of DNA with the sequence XXXX-ATGACTCAT-XXXX (and its complementary strand) and not bind to a mismatched DNA sequence (a sequence with one different pair of bases).

3. We will use competitive binding studies to show that our system site specifically binds to DNA and is not using general DNA binding phenomena.

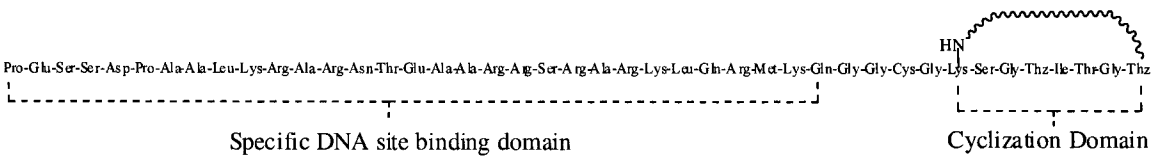

Figure 1 The liner peptide sequence including the metal binding and DNA binding domains

Background and Significance

The ability to selectively label DNA is an important part of utilizing the rapidly expanding genome library, as well as the development of the next generation of genetic sequencing methods.[3] The ability to label and screen DNA on a microchip scale[4] will dramatically lower costs and increase efficiency, as well as increase the sensitivity over current capillary electrophoresis methods.

The incorporation of redox active metal centers into oligonucleotides has recently been accomplished[5] by the addition of a ruthenium or osmium metal complex (Figure AA.2) to a triphosphate that can be incorporated into a growing nucleotide chain. This work shows promise, but has the significant drawbacks of: i. a complicated multi-step synthesis; ii. the labeled compound must be present when the nucleotide is synthesized; iii. the use of expensive organometallic precursors.

Figure 2A

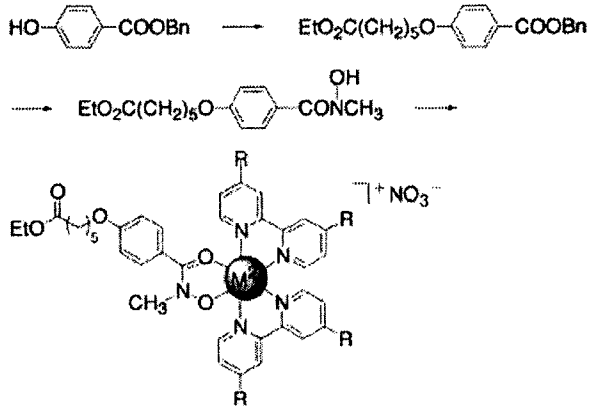

Figure 2B

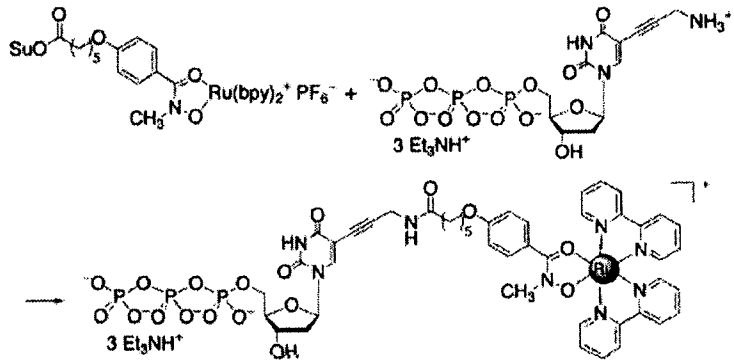

Figure AA.2A-B. The synthesis and nucleoside triphosphate incorporation procedures of Tor's redox active Ru compound (modified from Weizman, H.; Tor, Y *J. Am. Chem. Soc.* **2002**, *124,* 1569.)

The current proposal does not have any of these disadvantages, utilizing a combination of peptides with known synthetic structures. Both the metal chelating domain and the DNA binding domain are parts of the peptide. Inexpensive copper (II) nitrate will serve as the metal source, which can be incorporated during the cyclization of the metal chelating domain, or inserted after cyclization.

Cyclic peptides, and their ability to bind to metal ions, have been known for at least 60 years. Two of the earliest know cyclic peptides are the natural products actinomycin[6,7] and valinomycin,[8] both of which are involved in ion transport. The list of natural cyclic peptides has grown rapidly, and now includes lissoclinamide, raocyclamice, mollamide, cyclooxazoline, and ascidiacyclamide, all which were harvested from the marine environment.[9,10] It is thought that the purpose of these complexes may be to transport metal ions. Over the last 30 years, artificial cyclic peptides have been used as anti-fungal agents,[11] biologically active ligands,[12] in vivo metal reductants,[13] in vivo imaging agents,[14] ion and electron transporters,[15] and protein active site models.[16]

Cyclic peptides have several properties that make them ideal for our system. Peptides are easily synthesized, and may even be purchased commercially here at CSU.[17] The cyclization reactions of peptides have also been well studied,[1] and can be accomplished either with or without metal templates. The abilities of cyclic peptides to coordinate to metals (including lithium, sodium, potassium, cesium, cadmium, mercury, silver, zinc, and cobalt,[10] copper,[18] and iron,[19]) have been studied, and the x-ray crystal structures of several of the complexes have been determined. Cyclic peptides display

increased stability over linear peptides, and should be stable under all of the conditions that we will employ.

There is a solution structure of copper (II) coordinated to a cyclic peptide similar to the one we will use in our system (Figure AA.3).[18] It displays several features that will make it a good choice for our system. Comba and co-workers have demonstrated the ability to synthesize both a mono-copper and a di-copper compound, with the di-copper being more stable.

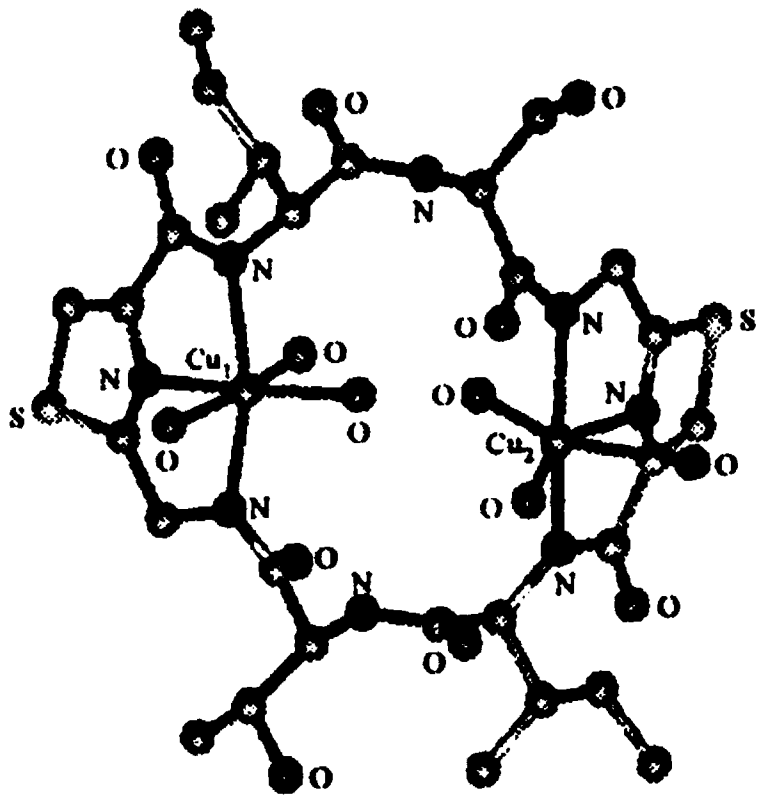

Figure AA.3. A molecular model of Comba's di-nuclear cyclic metal chelating peptide, using structural parameters based on EPR data (taken from Comba and co-workers, *Inorg. Chem.* **1998**, *37*, 6721.)

The mono-copper form has a LMCT transition in the UV region, and a d-d transition at 708 nm (in the presence of base). The di-copper form has UV-visible transitions at 590 nm and 540 nm. These wavelengths are all significantly different than the 220 nm and 320 nm absorbances of the metal free ligand giving us an optically active label in a region of the spectrum that should be free from interference. The spectra were also independent of the copper salt used, allowing us to use the readily available copper (II) nitrate. Using copper (II) also allows us to use other analytical methods of detection, (such as EPR, or radioactive methods using ^{67}Cu)[20] if the optical absorbance method isnot sufficiently sensitive at the required concentrations.

The metal chelating domain is formed through the cyclization of the χ amine side chain of the lysine. This allows us to synthesize the selective binding domain in a linear fashion along the α amine on the lysine (Figure AA.1). A glycine unit is used as a spacer,[21] which should allow the DNA binding domain to bind without any significant interference from the cyclic domain.

Protein containing molecules have demonstrated the ability to selectively bind to DNA. One of the earliest examples is the natural product bleomycin,[22] which has a metal binding peptide unit, a DNA intercalating di-thiazole unit, and a DNA binding sugar moiety. The DNA binding and reactivity associated with bleomycin has led it to be labeled a "treasure chest of bioinorganic chemistry."[22] The ability to bind DNA to peptides[23] and to find peptides that recognize DNA[24] is an important topic in biopharmaceutical chemistry.

Many sequence specific DNA binding peptides have been studied, most of which form an essential α helix-turn-α helix motif.[25-27] This motif is fundamentally important

in DNA binding specificity.[28] A "bZIP" motif has been discovered in transcriptional regulation proteins,[29] and their ability to bind DNA has been studied and modeled by McKnight and co-workers (Figure AA.4).[30]

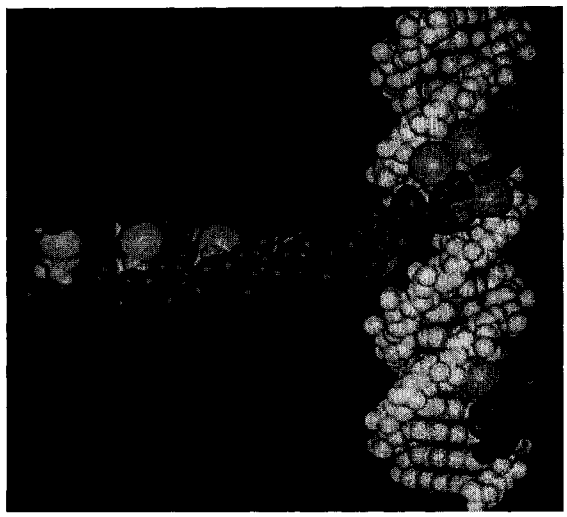

Figure AA.4. A model of a dimeric bZIP protein interacting with a 20-bp sequence of DNA (taken from Vinson, C. R.; Sigler, P. B.; McKnight, S. L. *Science*, **1989**, *246*, 911.)

The model shows the proper secondary structure as the peptide binds to the major grove of the DNA helix, as expected. The bZIP proteins have been shown to consist of two distinct regions: a basic region, and a leucine zipper region, which contains a Leu residue every seventh amino acid. Using this information, O'Neil and coworkers were able to synthesize a small peptide with the "bZIP" binding region, and were able to study its binding to DNA. Later, McKnight and co-workers[31] were able to show that specific binding could be accomplished with only the basic region of the "bZIP" motif, CNC4-br1

[a peptide with the amino acid sequence: (Pro-Glu-Ser-Ser-Asp-Pro-Ala-Ala-Leu-Lys-Arg-Ala-Arg-Asn-Thr-Glu-Ala-Ala-Arg-Arg-Ser-Arg-Ala-Arg-Lys-Leu-Gln-Arg-Met-Lys-Gln-Gly-Gly-Cys)], and that the two regions of the peptide were capable of functioning independently. Others have also studied similar peptides. Ebright and co-workers[32] have shown the use of a special branched "bZIP" motif with two basic regions and their specific binding to asymmetric DNA.

CNC4-br1 will be used as the DNA binding portion of our system for several reasons. It is 34 amino acids long, making is easy to synthesize yet large enough to overcome any non-specific binding caused by the metal chelating region of our molecule. It binds to DNA showing that it is still long enough to form the necessary α helix-turn-α helix structure, which should avoid interfering with the relatively planar chelating domain. Its binding to DNA has been studied extensively, and any necessary modifications to change DNA binding specificity should be easily made.

A significant advantage of our peptide system is that either domain can be changed without major changes in the overall synthetic methods. Our synthetic peptide system will allow us to screen for DNA with the specific sequence ATGACTCAT (and it's complementary strand). The nature of our system will then allow us to modify the DNA binding domain and screen for a different sequences. For example, we could screen for ATGACGCAAT (and its complementary strand) by synthesizing Ebright's peptide[32] or CACAATATATATTGTG (and its complementary strand) by synthesizing Grokhovsky's peptide[25] along with our chelating domain. Our system will also allow us to quantitate different peptides binding to any DNA sequences of interest. Currently,

studies of this type are very time consuming, relying on footprinting[33] methods, or on circular dichroism studies that can only quantitate the helical content of the protein.

Additionally, we could also change the chelating domain in a similar manner. If we wanted to change the metal ion in our system, we could use $(\text{-Glu-Sar-Gly-Gly})_2$[15], which has been shown to chelate calcium ions by Deber and co-workers, and attach it to our specific DNA binding domain.

There are also potential future applications of the complex in-vivo, as the cyclic peptides alone are already being used.[14] This could lead to a non-invasive way to look for a specific gene while leaving the DNA inside of a cell.

Research Design and Methods

The synthesis of the 43 amino acid linear peptide[34] should be easily attainable by standard Merrifield solid-phase synthesis[35] on polystyrene. Starting at the C-terminus of the metal-binding domain, the first seven amino acids can be added by standard procedures. An important step in the procedure would be the protection of the χ amine of lysine (the eighth amino acid from the C-terminus) keeping it free for the cyclization reaction. Protection of the χ amine with t-butyloxycarbonyl should be sufficient. An alternative method available to us would be the purchase of the linear peptide.[17]

The cyclization reaction can be accomplished in a similar manner to the cyclic peptide synthesis of Comba and co-workers.[18] Dissolution of the peptide in dimethyl formamide followed by the addition of diisopropylethylamine caused the cyclization of their peptide to occur in one hour. Protecting groups may be needed to suppress unwanted cyclization reactions. Preparatory HPLC purification followed by addition of two equivalents of copper nitrate should complete the synthesis. We will also investigate the effect of adding copper (II) nitrate to the cyclization reaction. There may be a templating effect that could increase the yield of the desired product.

If Comba's cyclization method does not work, Kenner's "safety-catch linker" method[1,2] (Figure AA.5) will be tried. In this method, a cyanomethylsulfonamide is inserted between the support and the first amino acid. The attacking amine in our case would be the χ amine on lysine-8.

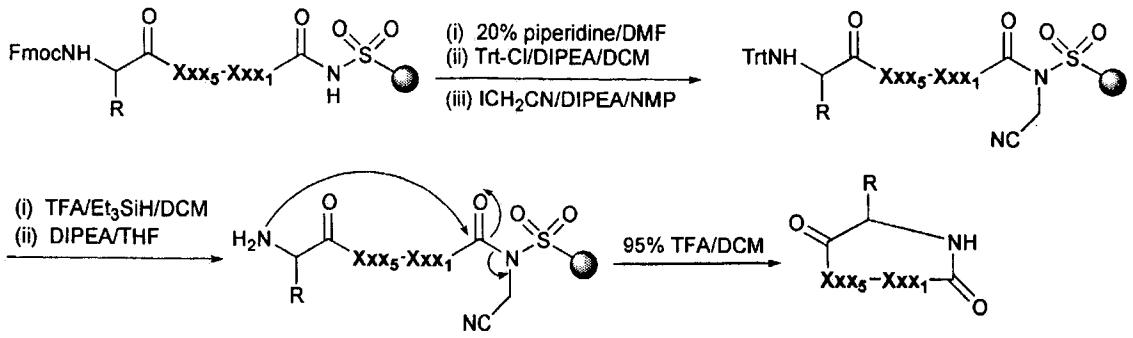

Figure AA.5. A schematic diagram of a general cyclization reaction using Kenner's "safety-catch linker" mechanism (taken from Lambert. J. N.; Mitchell, J. P.; Roberts, K. D. *J. Chem. Soc. Perkin, Trans. 1*, **2001**, 471.)

With the binding molecule synthesized, an assay can be performed (Figure AA.6). DNA of the sequence XXXXXXXXXX-ATGACTCAT-XXXXXXXXXX (and its complementary strand) can be synthesized and left on the support. Our binding peptide can then be added and allowed to react. The support can then be washed[36] and an absorbance (or a different quantitative method) can be used on the copper (II) that is bound to the peptide-DNA complex. The experiment can be performed over a variety of concentration ranges and temperatures, and the binding affinity for the protein to DNA can be calculated. The prediction for this experiment is significant binding, at μM peptide concentrations. An important control experiment here involves doing a standard footprinting[33] experiment to ensure that the peptide is binding at the DNA site of interest.

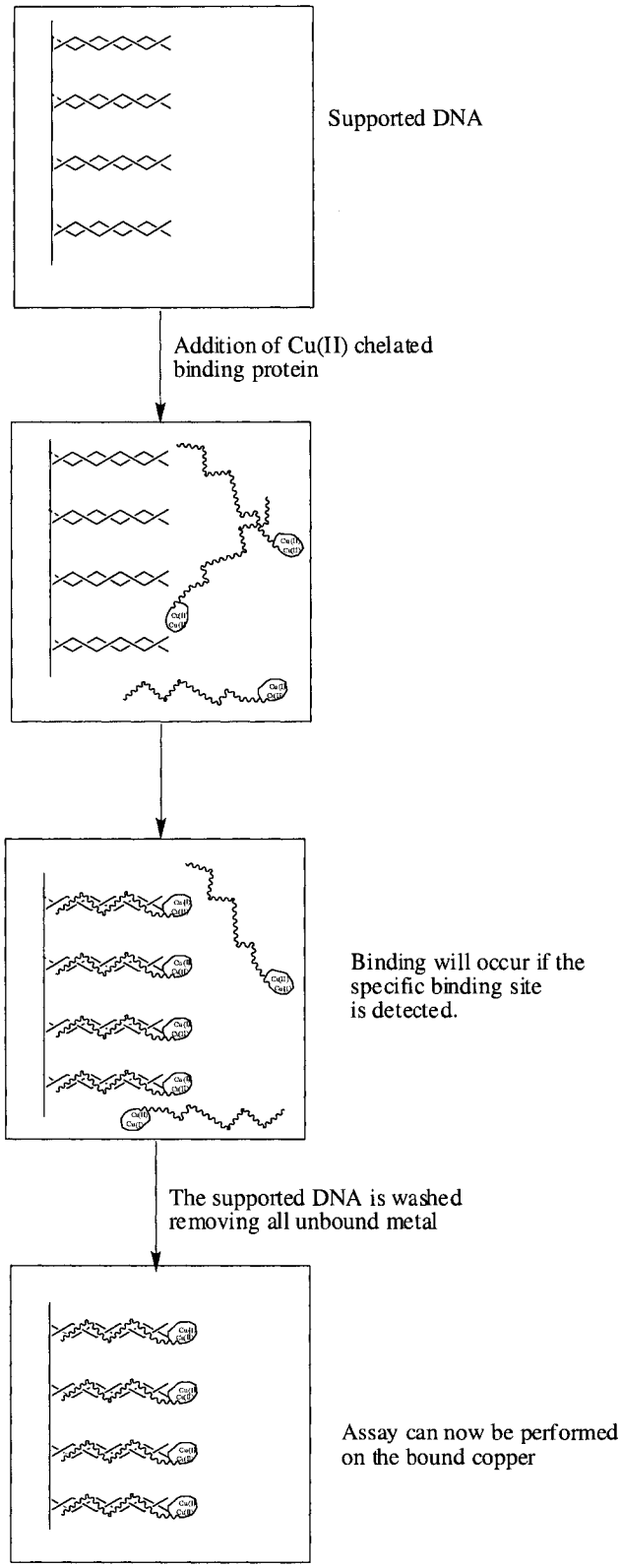

Figure 6. A schematic design of a DNA binding experiment using metal chelated binding peptide.

The next assay should be on a strand of DNA with a one different base than the ATGACTCAT sequence. The prediction for this experiment is a dramatic decrease in binding affinity. This experiment also serves as another control experiment testing for non-specific binding. If there is significant binding, a third assay with two or more mismatched bases will be necessary.

Competition experiments (Figure AA.7) can be set up, with supported DNA with the ATGACTCAT sequence and flowing exogenous DNA with a mismatched sequence. The relative affinity can then be tested by determining if the copper remains bound to the supported DNA, or if it flows through with the exogenous DNA. The complementary experiment is also of interest, and a comparison between the two will show if there is a binding advantage caused by the support.

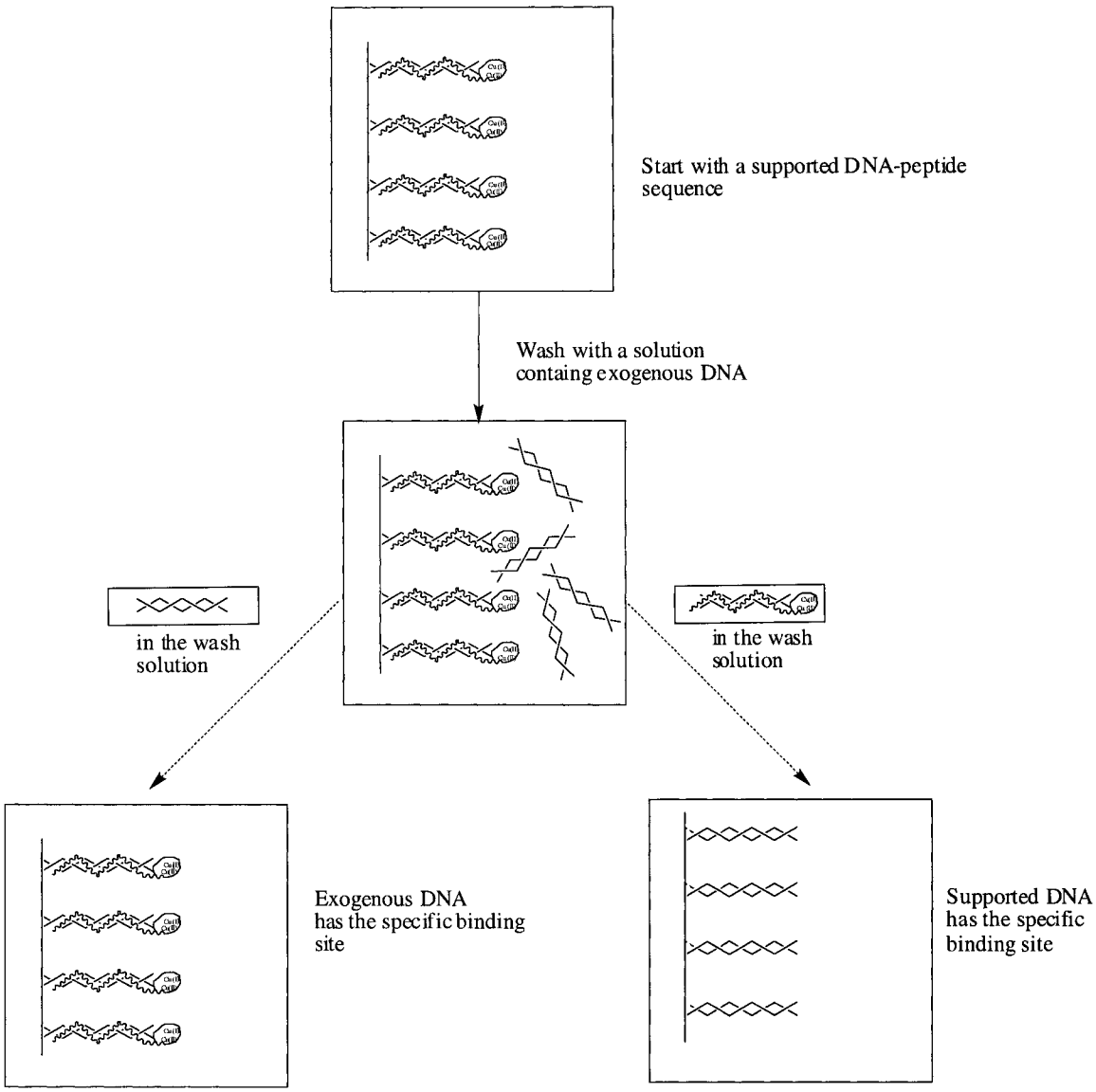

Figure 7. A schematic design of a DNA competition experiment

The greatest potential for our system is that it should be applicable to any binding peptide that is discovered. In order to recognize a specific site on a chromosome, a binder has to be able to bind to a site with 16 base pairs, and be able to recognize a single mismatch. In order to find such a molecule, there must be a method of rapid screening. Once developed, this method offers a simple and reliable way to help screen the large number of DNA binding proteins that are surely being developed right now.

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[20] The $t_{1/2}$ of ^{67}Cu is 61.88 hours. This should allow for its use in radiolableing experiments that can be accomplished on a short timescale.
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[33] In a footprinting experiment, the DNA is labeled at one end, and after binding, the DNA is cleaved nonspecifically. Looking at the fragmentation on a gel electrophoresis experiment then allows the experimentalist to determine where the DNA was not cleaved, and assume that binding is taking place on that part of the DNA.
[34] High purity peptides of 102 amino acids have been synthesized by Grokhovsky and co-workers,[25]on standard phenylacetoamidomethyl (PAM) resin.

[35] Fox, M. A.; Whitesell, J. K. Organic Chemistry 2nd Edition Jones and Bartlett Publishers: Boston MA **1994.**

[36] A metal chelating agent such as EDTA can be used in the wash solution to ensure complete removal of all except specifically bound metal.

APPENDIX B

GENERAL STATEMENT ON "JOURNALS-FORMAT" THESES

(Written by Professor Richard G. Finke)

The Graduate School at Colorado State University allows, and the Finke Group in particular encourages, so-called journals-format theses. Journals-format theses, such as the present one, consist of a student written and lightly edited literature background section, chapters corresponding (in the limiting, ideal case) to final-form papers either accepted or at least submitted for publication, a summary or conclusions chapter, and short bridge or transition sections between the chapters as needed to make the thesis cohesive and understandable to the reader. The "bridge" sections and summary are crucial so that the thesis fulfills the requirement that the thesis be an entity (an official requirement of most Graduate Schools). All chapters (manuscripts) in a journals-format thesis must of course be written initially by the student, with subsequent (ideally light) editing by the Professor, the student's committee, and even the student's colleagues where appropriate and productive.

The advantages for doing a journals-format thesis are several-fold and compelling. Specifically, some of the major advantages are: the level of science (i.e. of refereed, accepted publications) is at the highest level; the student and Professor must interact closely and vigorously (i.e. to bring both the science and the writing to their highest level), hence the student is getting the best education possible and is being at least exposed to (if not held to) the highest standards; the needed clean-up or control experiments that invariably come

up have all been identified and completed before the student leaves; there are no further time demands once the student has left the University (since all the publications are at least submitted; it is terribly inefficient to try to complete either writing or often specialized experiments once the student has left); and the American tax payers, who ultimately pay the bill for the research, are getting their money's worth since all the research is published and thus widely disseminated in the highest form, as refereed science. Professorial experience teaches that a student who has achieved a journals-format thesis has indeed received a better education and has learned critical thinking and clear writing skills that will serve them well for a lifetime.

Experience also teaches, however, that much more than light editing is often needed in at least some student theses; it follows, then, that considerable professorial writing and editing might be needed for at least the initial chapters of most journals-format thesis. Indeed, a journals-format thesis is not recommended (and may not even be possible) for less strong students. Hence, the issue arises of exactly how much of the science and the writing, in the final (or submittable) chapters, is due to the student vs. the Professor and whether or not this level of contribution constitutes that acceptable of a new Ph.D. and independent investigator. An additional potential problem is that the journals-format thesis does not include unpublished/unpublishable experimental results (i.e., unless they are in footnotes or the Supporting Information), which may have significant value for future members of the research group.

To deal with these issues, several recommendations are made; the recommendations below have been discussed with the committee signing Kenneth M. Doll's dissertation. (Mr. Doll's dissertation is the tenth such thesis from the Finke Group following Dr. C. Garr's, Dr. Y. Lin's, Dr. M. Pohl's, Dr. J. Sirovatka,'s, Dr. J. Aiken's, Dr. R. Suto's, Dr. J. Widegren's dissertations, and Ms. K. Weddle's and Mr. W. White's Masters theses.) The recommendations are:

(i) That the present pages be enclosed in the thesis until such a time as it is no longer needed;

(ii) That for each chapter it is detailed, and to the satisfaction of the committee and the advisor, who made what contributions, both of intellectual substance and writing. [Substantial contributions of other students or Professors should of course also be acknowledged. In the case of disagreements, the various drafts (i.e. as their electronic files) can be examined by the committee (in light of a knowledge of who wrote which draft) to easily determine who contributed what. In possible borderline or controversial cases it may even be advisable to keep all (electronic) drafts of the papers as a record];

(iii) That it be specifically stated whether or not all the experimental work is the Ph.D. candidate's;

(iv) Furthermore, it is recommended that allowances be made for the expectation that a greater degree of involvement of the professorial advisor is likely in a journals-format thesis than in a traditional thesis. [That this is reasonable follows from the fact that some Professors write 100% of all their papers; this, unfortunately, robs the student of the valuable experience of participating in the science and the end product as practiced at the highest levels.];

(v) Notwithstanding (iv), there needs to be ideally no more than ca. 40% Professorial writing contribution in a given *early* chapter in the thesis, and there should be a clear evolution in the thesis of a decreasing professorial involvement to, say, a 10-20% direct contribution in the last chapter or two;

(vi) As a further aid towards separating out the candidate's and the professorial (and other) contributions, it is recommended that the Introductory (usually literature background) chapters(s) and at least the final chapter be lightly edited only, so that authentic examples of the student's contributions are documented in an unambiguous form.

(vii) In order to avoid the loss of useful, but unpublished/unpublishable, experimental work by the student writing a journals format-thesis, the Finke Group

requires the following: (1) carefully kept laboratory notebooks; (2) mandatory research reports detailing the results of any unpublished work; and (3) the extensive use of Supporting Information and textual footnotes, where appropriate, in all published work.

APPENDIX C: % CONTRIBUTIONS

CHAPTER	EXPERIMENTS	WRITING
CHAPTER 1 INTRODUCTIONS AND REVIEW OF RELEVANT LITERATURE	NA	KMD 95% RGF 5%
CHAPTER 2 THE SYNTHESIS AND CHARACTERIZATION OF 8-METHOXY-5'-DEOXYADENOSYLCOBALAMIN	KMD 100%	KMD 70% RGF 15% PEF 15%
CHAPTER 3 THE FIRST EXPERIMENTAL TEST OF THE HYPOTHESIS THAT ENZYMES HAVE EVOLVED TO ENHANCE QUANTUM MECHANICAL TUNNELING	KMD 87% BRB 13%	KMD 70% RGF 15% BRB 15%
CHAPTER 4 A RARE EXPERIMENTAL TEST OF THE HYPOTHESIS THAT ENZYMES HAVE EVOLVED TO ENHANCE QUANTUM MECHANICAL TUNNELING IN HYDROGEN TRANSFER REACTIONS: THE β-NEOPENTYLCOBALAMIN SYSTEM	KMD 100%	KMD 90% RGF 10%
CHAPTER 5 SUMMARY	NA	KMD 95% RGF 5%
APPENDIX A EFFICIENTLY LABELING DNA BY COMBINING CYCLIC PEPTIDE COPPER (II) CHELATING ABILITY AND DNA SITE SELECTIVE BINDING ON A SINGLE PEPTIDE STRAND	NA	KMD 100%
APPENDIX D A RE-INVESTIGATION OF THE AXIAL BASE EFFECT IN COENZYME B_{12}-DEPENDENT CHEMICAL PRECEDENT REACTIONS	KMD 100%	KMD 75% RGF 25%

APPENDIX D

Adenosylcobinamide Plus Exogenous, Sterically Hindered, Putative Axial-Bases: A Re-investigation Into the Cause of Record Levels of Co-C Heterolysis

Kenneth M. Doll and Richard G. Finke*

Contribution from the Department of Chemistry, Colorado State University

Fort Collins, Colorado 80523

Intended for submission to Inorganic Chemistry.

This chapter is intended for submission to *Inorganic Chemistry* with only modifications to the margins and figure numbering for compliance with that journal's requirements. This chapter contains a detailed account of the re-investigation of the thermolysis reaction of $AdoCbi^{+}BF_4^{-}$ plus exogenous axial-base in ethylene glycol solution. The goal of these studies is to verify or refute the hypothesis that both σ and π effects of the added axial-base are important to the mode of Co–C bond cleavage (biological homolysis vs abiological heterolysis).[1] The first alternative hypothesis tested herein is that a possible low-level impurity in the added axial base is causing the observed Co–C bond cleavage products and rates. If true, this would invalidate earlier conclusions based on a previous thesis.[2]

To test this low-level impurity hypothesis, a detailed kinetic and product study of the thermolysis reaction of $AdoCbi^{+}BF_4^{-}$ with unpurified and with carefully purified bases has been carried out. The results *reveal no statistically significant change within experimental error* in comparison to the results in an earlier thesis and resultant paper.[1,2] The important conclusions in those publications are, therefore, not the result of impurities in the exogenous bases.[1,2]

However, in light of the experimental problems in a prior thesis (Sirovatka, J. M. Ph.D. Thesis. Colorado State University: Fort Collins, 1999),vida infra, we decided to re-investigate the leading alternative hypothesis to completing σ and π effects which we had considered before,[1,3] namely that: "deprotonation of ethylene glycol by 1,2-Me_2-Im yields $HOCH_2CH_2O^{-}$, and that strongly σ-donating species is responsible for the observed Co–C heterolysis".[1,3] This "ethylene glycolate anion effect" hypothesis[1,2] has general precedent in the increased Co–C bond heterolysis caused by strong sigma donors such as

CN^-.[4] Earlier investigations[1,3,5] of this hypothesis involved control experiments investigating in the thermolysis of $AdoCbi^+BF_4^-$ in which Proton Sponge™ [1,8-bis(dimethylamino)napthaline] was added to produce ethylene glycolate anion and the conjugate acid of Proton Sponge™, PS™-H^+. Herein, we repeated such control experiments with a variety of bases over a more varied concentration range leading to the discovery of a linear correlation between the calculated initial ethylene glycolate anion concentration and the percentage of observed Co–C bond heterolysis. Additionally, control experiments with *sodium* ethylene glycolate has led to the discovery of a previously unappreciated, important cation effect on Co–C heterolysis. This cation / proton effect, expected from the established mechanism of Co–C heterolysis in AdoCbl,[6] along with ethylene glycolate anion is the *simplest* explanation for the observed Co–C bond cleavage in $AdoCbi^+$ with added bases in ethylene glycol.

Other experiments found fault in another, albeit minor, portion of the earlier thesis work[1-3] in which Co(II)Cbi^+ was titrated with the axial bases. Contrary to Sirovatka's earlier report,[1-3] herein it is found that bases which are sterically hindered near the coordinating nitrogen, such as 2,6-dimethyl pyridine and 1,2-dimethyl imidazole, do *not* detectably bind to Co(II)Cbi^+.

This chapter does not pertain to QM tunneling or Klinman's hypothesis. Hence, it has been placed in this appendix consistent with it being an entirely separate piece of science. It is important, however, in that it brings to a definitive closing point a controversy that had existed in the B_{12} literature.

Abstract

A recent paper (Trommel, J. S.; Warncke, K.; Marzilli, L. G. *J. Am. Chem. Soc.* **2001**, *123*, 3358) has claimed that "Artifacts caused by such impurities (i.e., in the exogenous, putatively axial bases) have also confounded results of model experiments devoted to assessing the mechanism of the Co–C bond cleavage step" of $AdoCbi^+$ in the presence of those exogenous bases in ethylene glycol solvent. That paper went on to claim that "We attribute the reported observations to impurities in the two (i.e., 2-Me-pyridine and 2,6-Me_2-pyridine) ligands". The work in question is from a thesis (Sirovatka, J. M. Ph. D. Thesis. Colorado State University: Fort Collins, 1999) that was then published in part elsewhere (Sirovatka, J. M.; Finke, R. G. *Inorg. Chem.* **1999**, *38*, 1697). An immediately obvious problem in the logic of the criticism is that the authors of that criticism (*op. cit.*) did not perform any *kinetic studies* nor did they examine *imidazole ligands*, the source of the data which led to our earlier conclusions (Sirovatka, Finke, *op. cit.*). Nor did the authors of the criticism consider any alternative hypotheses besides their "impurity hypothesis". Hence, their very strongly worded assertions that our conclusions were "in serious doubt" or "cannot be accepted as being correct" and needed to be "withdrawn" are not based on a firm foundation or scientific method. Nevertheless, Marzilli and co-workers' report did find several problems with other prior literature as well as experimental problems with a prior thesis from our group (J. Sirovatka, *op. cit.*). Hence, herein we have undertaken a reinvestigation of the thermolysis reaction of $AdoCbi^+BF_4^-$ in ethylene glycol solution with the exogenous bases, N-methylimidazole (N-Me-Im), 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).

Multiple purities of each base have been utilized as a check to see if, as others have claimed, impurities are causing the observed homolysis and heterolysis product distributions. The answer is that the "impurity hypothesis" is disproven: 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 displays an invariant ~ 16 ± 5 % heterolysis as a function of different purification methods. Overall, these experimental results and those cited below are definitive in refuting the claims of others (*op. cit.*) that a low level impurity in the bases is the source of the observed Co–C cleavage products for $AdoCbi^+$ thermolysis in ethylene glycol with exogenous bases. The report of others (*op. cit.*) is, however, invaluable in showing: that impurities *are* in hindered pyridines such as 2-Me-pyridine, that carefully purified 2-Me-pyridine does *not* bind to $Co(II)Cbi^+$, and, therefore, that $Co(II)Cbi^+$ 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-visible spectroscopy (J. Sirovatka, Ph.D Thesis, *op. cit.*). In light of the experimental problems in the aforementioned thesis, we have revisited our earlier, four alternative hypotheses for the unexpectedly high amounts of Co–C heterolysis in the $AdoCbi^+$ plus sterically bulky bases. Specifically we show herein that our prior #1 alternative hypothesis is in fact correct: that the added bases simply deprotonate the ethylene glycol solvent, forming ethylene glycolate anion *and Base-H*$^+$, and that the [Base-H^+] [$HOCH_2CH_2O^-$] combination—not an unspecified impurity in the axial bases—is the key agent behind the previously ill-understood Co-C heterolyses. Also reported are $Co(II)Cbi^+$ titrations with four bases (1,2-Me_2-Im, N-Me-Im, pyridine, and 1,2-Me_2-py). These experiments

confirm Marzilli and co-workers (*op. cit.*) correction of the earlier thesis work (*op. cit.*) by showing that carefully purified, sterically hindered bases do *not* bind to Co(II)Cbi^+. Finally, our current best synthesis and purification of $AdoCbi^+BF_4^-$ is presented in the Supporting Information, work that updates our 1987 synthesis of $AdoCbi^+BF_4^-$ (Hay, B. P.; Finke, R. G. *J. Am. Chem. Soc.* **1987**, *109*, 8012).

Introduction

Adenosyl*cobalamin* (AdoCbl) is an essential cofactor essential for at least 17 different enzymatic systems.[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] show a remarkable ~10^{12} fold acceleration of the cleavage of this bond. Exactly how AdoCbl-dependent enzymes accomplish this rate acceleration remains a mystery, however.[13,14]

Adenosyl*cobinamide* (Figure AD.1) ($AdoCbi^+$) is an analog of AdoCbl where the α-axial 1,2-Me_2-benzimidazole ligand (on the lower side of the corrin ring), has been removed.

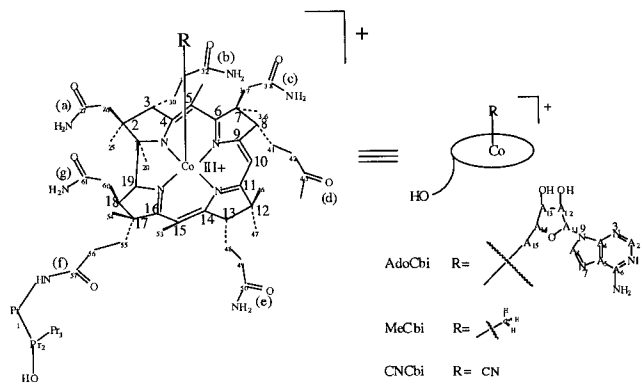


Figure AD.1

The structure of an alkyl cobinamide following IUPAC nomenclature.[15]

Studies of this molecule (and its binding to exogenous bases (vide infra))[1-3,5,16-18] have been shown to be biologically relevant via three crystal structures: two structures of 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 1,2-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.

A controversial and confusing aspect coenzyme B_{12} chemistry has been the precise Co–N(histidine) axial bond length in the AdoCbl•enzyme complexes. The structures show a 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 ligand states in the crystals thwart clear conclusions".[23] 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 analogs 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 4th or 5th shells on other (non-B_{12}) systems.[28-31] EPR studies of the MMCoA•B_{12} holoenzyme find a hyperfine coupling constant of 108 Gauss, consistent

with a normal length Co–N bond,[24,32] and Marzilli and co-workers' most recent studies are quite important in also supporting a normal-length Co–N axial base bond.[23]

However, a lengthened Co–N bond could still be involved in transition state structure for Co–C cleavage; hence, a variable Co–N bond length has been postulated to be important to Co–C bond cleavage[33,34] through both steric[35,36] and electronic[37,38] effects. An early hypothesis which grew out of the *apparently* long Co–N(histidine) bond length is that the enzyme might be using the 1,2-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] even if primarily via 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 ~10^2 times less reactive than AdoCbl; hence, the axial ligand is *not* the source of the enzymes 10^{12} fold acceleration of the Co–C bond cleavage. This early, important result and conclusion, one under appreciated perhaps even now, has withstood the test of time.[36,41-48] 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. Solutions of $AdoCbi^+$ were studied with a series of pyridines[16] and imidazoles;[5] the equilibrium binding constants (K_{assoc}), bond activation parameters ($\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values), and the reaction products (percent homolysis vs percent heterolysis) were obtained. Within the series of pyridines, the stronger σ donors gave the expected stronger ΔH, higher K_{assoc}, and more heterolysis (for example: pyridine, $pK_a = 5.2$, $\Delta H = -3.3 \pm 0.4$ kcal mol^{-1}, $K_{assoc} = 1.0 \pm 0.2\ M^{-1}$, 7 % heterolysis; p-$Me_2N$-pyridine, $pK_a = 9.7$, $\Delta H = -6.5 \pm 1.0$ kcal mol^{-1}, $K_{assoc} = 2.5 \pm 0.2\ M^{-1}$, 45 % heterolysis). A comparison of N-methylimidazole (N-Me-Im)[17] vs 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, ΔH -7.8 ± 0.4, and as much heterolysis, 48 %, as the more basic 4-Me_2N-pyridine ($\Delta pKa = 2.4$ units more basic than N-Me-Im). Deconvolution of the kinetic data also revealed that $AdoCbi^+$ plus N-Me-Im undergoes Co–C *heterolysis* 30,700 fold faster than does $AdoCbi^+$ 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] The several hypotheses for how B_{12}-dependent enzyme can limit Co–C heterolysis have been previously discussed: limitation of proton access[49] (required for protonation of the adenine anion eventually produced by Co-Ado heterolysis); conformational restriction away from the heterolysis-favoring trans-antiplanar Co-C_α-C_β-O arrangement;[50] or a long

Co–N bond, predicted by MO calculations on B_{12}-models to favor homolysis at Co–N ~ 2.4 Å.[39]

We previously tested the long Co–N hypothesis via molecular modeling, kinetic and product studies of axial-bases of increasing steric hindrance (Figure AD.2) and concomitantly longer Co–N bond.[1,51] Molecular modeling 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. 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 $[AdoCbi•\textit{solvent}]^+$ shows no tendency to bind bulky bases at its axial coordination position, probably due to the estimated 8 kcal/mol Co-solvent bond energy that binding of other axial bases must overcome.

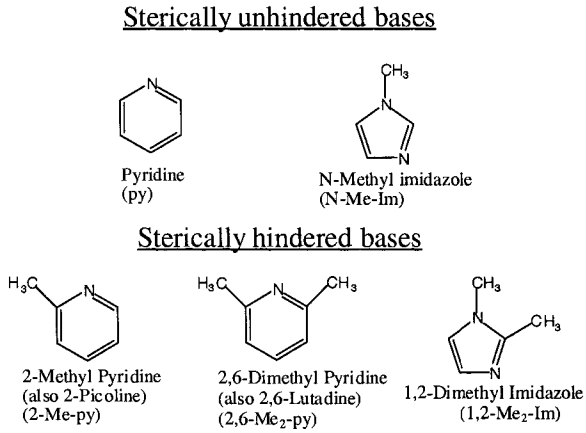


Figure AD.2

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

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] as well as herein, *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 (0.39 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 detailed 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. Five possible 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 one-half of 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)napthaline], showed only 5% Co-heterolysis (p. 20 of the Supporting Materials[1])—a far cry from the 91 % heterolysis seen with added 1,2-Me_2-Im. That (as we will see misleading) control experiment *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. Specifically, we worried that the thermolysis of 1 x 10^{-4} M AdoCbi$^+BF_4^-$ with high amounts of, in some cases, very poorly coordinating axial bases (0.3 M; 3000 fold excesses). These experiments require 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. This issue was not taken as seriously as it should have been[2] except to check the purity of the 1,2-Me_2-Im by NMR (see p. 1705 and 1706 elsewhere, top right-hand column). The "insidious impurity issue"[52,53] eventually became de-emphasized as we struggled to understand the puzzling 1,2-Me_2-Im results, which eventually focused us 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^+ counter cation 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 Co–C heterolysis than do the Base-H^+ counter cations (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] New titration experiments, performed with both purified and unpurified bases, are reported herein (Supporting Information Figures AD.S1-AD.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$^+$. These Co(II)Cbi$^+$ plus sterically hindered base studies were initially only done *as extra control experiments* to see if we could obtain direct 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 irreproducible[2] 5% heterolysis in

the control experiment with [Proton Sponge-H^+][$HOCH_2CH_2O^-$] (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 the new experimental work, by another experimentalist (K.M. Doll), reported herein—despite the valuable report of others 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 a 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[64,65,66] have been used, the most common and also most practical literature purification[67,68-70] 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 by-product. Literature on the purification of sterically hindered pyridines dates back to the 1950s.[73-75] 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 be effectively 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 or not (impurity) binding to Co(II)Cbi^+ can be detected by EPR and UV-visible 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 others have 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, 1,2-Me_2-py, it was possible to use the *exact same bottles* that were used in the previous report.[1]

As was done in the previous experiments,[1] ethylene glycol solutions of AdoCbi$^+BF_4^-$ (1 x 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 nine different thermolysis solutions utilizing imidazoles, and fourteen different thermolysis solutions utilizing pyridines, shows that, *regardless of source or purity, the results did not change within experimental error from those we reported previously*,[1] Table AD.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 AD.S1 and AD.S2.

Table AD.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]

	% Heterolysis Previous Study[1]	% Heterolysis This Work	k_{obs} (s^{-1}) Previous Study[1]	k_{obs} (s^{-1}) This Work
N-Me-Im	48	52 ± 10	3.4 (± 0.2) x 10^{-5}	4 (± 1) x 10^{-5}
1,2-Me_2-Im	91	83 ± 7	4.3 (± 0.3) x 10^{-5}	4 (± 1) x 10^{-5}
2-Me-py	24	17 ± 5	1.0 (± 0.1) x 10^{-5}	1.1 (± 0.1) x 10^{-5}
2,6-Me_2-py	6	16 ± 5	0.89 (± 0.05) x 10^{-5}	1.0 (± 0.1) x 10^{-5}
None	2	5	0.32 (± 0.10) x 10^{-5}	Not Examined

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 $[(A_\infty/(A_\infty - A_t)]$ vs time plot at 474 nm, is 4(±1) x 10^{-5} s^{-1}, also within experimental error of the values in the original report, 3.4 (± 0.2) x 10^{-5} s^{-1}. Note that the k_{obs} rate constant has both homolysis and heterolysis contributions as Scheme AD.1 makes apparent and as the kinetic derivation elsewhere[1,16] and in the Supporting Information accompanying this paper (Section AD.S-1) make

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

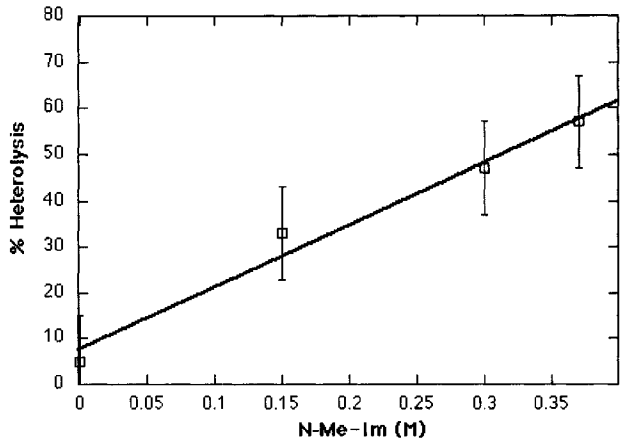


Figure AD.3 A plot of N-Me-Im concentration vs the observed percentage of $AdoCbi^{+}$ Co–C bond heterolysis. The slope and intercept of the line are 140 ± 15 % M^{-1} and 8 ± 4 % respectively.

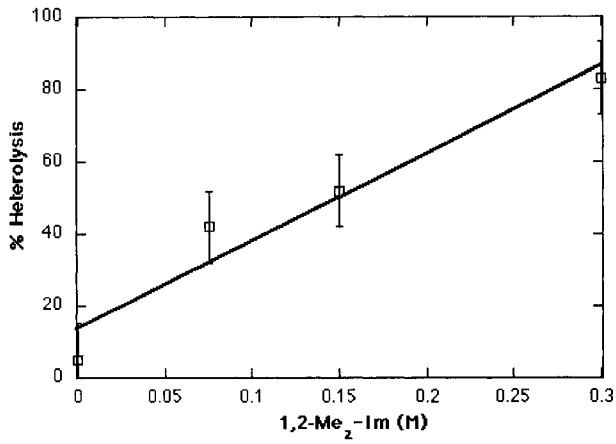


Figure AD.4 A plot of 1,2-Me_2-Im concentration vs the observed percentage of $AdoCbi^+$ Co–C bond heterolysis. The slope and intercept of the line are 240 ± 40 % M^{-1} and 14 ± 8 % respectively.

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 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 x 10^{-5} s^{-1}. In short, the data do not support the hypothesis that "an impurity is causing the Co–C observed heterolysis".[23]

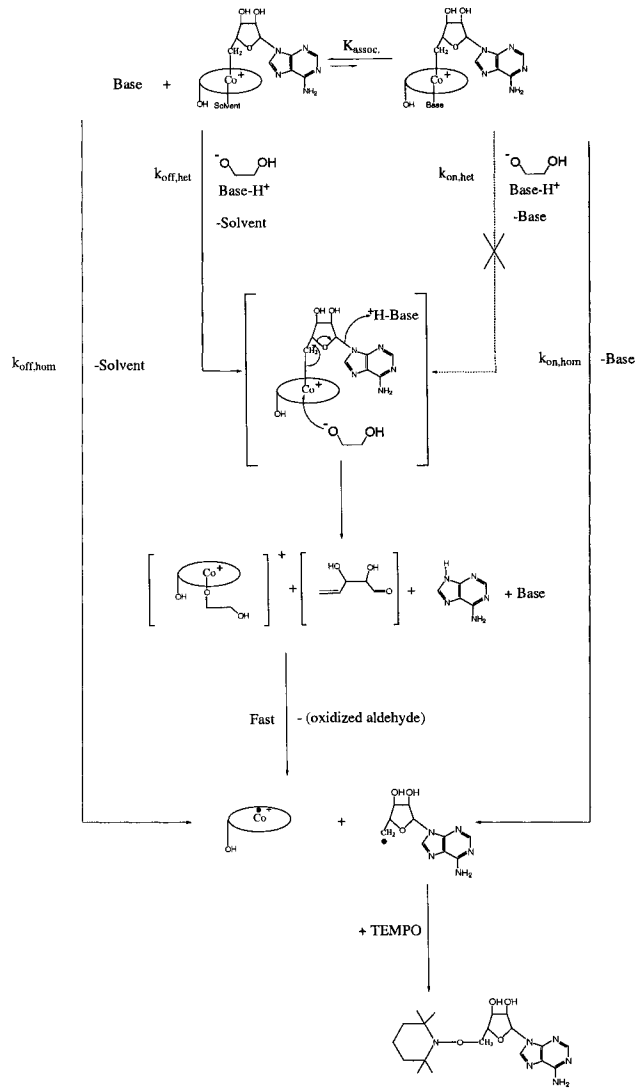
Control Thermolysis of Ado*Cbl* with Added Bases

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 equivalents 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 $1.8 \pm 0.2 \times 10^{-4}$ s^{-1} was observed for each reaction, a value that was within experimental error of a control thermolysis experiment performed concurrently using AdoCbl without these added bases. A value which is also within experimental error to literature values[6,9] (Table AD.S3). Again, no evidence for any effect of trace impurities in the axial base was found.

A Re-investigation 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 to, by a consideration of the known mechanism of Co–C heterolysis,[6] 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. This established mechanism, Scheme AD.1, makes apparent that *both* the glycolate anion,

[$HOCH_2CH_2O^-$], *and* the conjugate acid of the Base, [Base-H^+], can play a role in accelerating Co–C heterolysis. This then lead us to realize that the control we had done with Proton Sponge™, as a sterically bulky base (and thus non-coordinating and, before, seemingly ideal base to generate the [$HOCH_2CH_2O^-$]), may have been misleading. Of course, the attraction of the "glycolate anion" hypothesis all along[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 non-kinetically 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 full rate law for Scheme AD.1 is available in the Supporting Information (Section AD.S-1).



Scheme AD.1 The established reaction scheme[6,16] for the homolysis and heterolysis reactions of AdoCbi$^+$ in ethylene glycol and in the presence of TEMPO, including the important effect of [Base-H$^+$][glycolate$^-$] on the Co–C bond heterolysis pathway. The five key constants are defined by this scheme: K_{assoc}, $k_{off,hom}$, $k_{off,het}$, $k_{on,hom}$, and $k_{on,het}$. Our kinetic evidence (vide infra) requires that the ethylene glycolate anion attack, and protonation by Base-H$^+$, both occur before the rate-limiting step of the Co-C heterolysis reaction; our evidence does not detail the timing of these steps, however. Based on

literature precedent (Brown and other's seminal work cited in references 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 elswehere[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.

We tested 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 pKas 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 spans an initial [Base-H^+][$HOCH_2CH_2O^-$] concentration range from 1 x 10^{-4} M to 8 x 10^{-4} M. A plot of the percentage Co–C heterolysis vs [Base-H^+][$HOCH_2CH_2O^-$] *shows a linear dependence*, Figure AD.5. *The fact that data from all of the bases fit the same line is a very important observation when combined with the facts 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 AD.5 provide very strong evidence that the amount of [Base-H^+][$HOCH_2CH_2O^-$] is the Co–C heterolysis-causing agent.

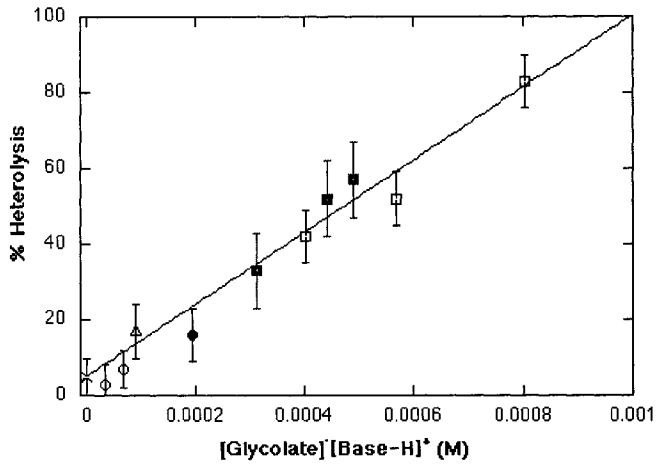


Figure AD.5 A plot of the percentage heterolysis of the Co–C bond vs the initial ethylene glycolate anion concentration calculated from pKa values and concentration of the bases used. The data points are for the bases: 1,2-Me_2-Im (□), N-Me-Im (■), 2-6-Me_2-py (●), 2-Me-py (△), pyridine (○), and no added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

We also repeated our earlier experiment[1] in which Proton Sponge™ was used to generate [Proton Sponge™-H^+][$HOCH_2CH_2O^-$] at 8 x 10^{-4} M, the level expected from the pKa 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 28 ± 8 % percent $AdoCbi^+$ 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^{TM}-H^+][$HOCH_2CH_2O^-$], and a linear correlation of the % heterolysis vs the concentration of [Proton Sponge^{TM}-H^+][$HOCH_2CH_2O^-$] is observed (Figure AD.S8 of the Supporting Information). Importantly, these results confirm the validity of the 28 ± 8 % percent Co–C heterolysis at 8 x 10^{-4} M [Proton Sponge^{TM}-H^+][$HOCH_2CH_2O^-$]. Furthermore, independent data from our first, 1996 report[16] also using [Proton Sponge^{TM}-H^+][$HOCH_2CH_2O^-$] fits nicely to the observed line, thereby providing additional confirmation of these now repeatable control experiments using Proton Sponge^{TM}.

In comparison to the results for the other Base-H^+ or Na^+ cations (vide infra), the [Proton Sponge^{TM}-H^+][$HOCH_2CH_2O^-$] system demonstrates the importance of the specific [Base-H^+] or other counter cation in the Co–C heterolysis process. Noteworthy is that the observed 28 ± 8 % for [Proton Sponge^{TM}-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 pKas of the [Base-H^+] species involved. All of the bases plotted in Figure AD.5 have a pKa value between 5.3 (pyridine) and 7.8 (1,2-Me_2-Im).[79] However, Proton Sponge™ (conjugate acid pKa 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[81,82,83] is expected *and observed.*

Interestingly, the observed 52 % heterolyis vs the calculated initial [Base-H^+][$HOCH_2CH_2O^-$] of 6.8 x 10^{-3} M in our 1996 report[16] studying AdoCbi+ with *Me_2-N-py* (conjugate acid pKa[84] = 9.7) also fits on the Proton Sponge^{TM} line and correlation already discussed (Figure AD.S8 in the Supporting Information). Since the protonation

site in Me_2-N-py is at the (less hindered) pyridine nitrogen, that is, Me_2-N-py-H^+,[79,85] this would seem to say that it is the pKa of the conjugate acid of the added base, and not the level of steric bulk about the added base's protonation site, that is crucial in the Co–C heterolysis process.

Further evidence for a counter cation 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 in between $[Na^+ \bullet HOCH_2CH_2O^-]$ and percentage heterolysis of the Co–C bond in this system (Figure AD.S.8 in the Supporting Information). A comparison of the slope of the line for Na^+ as the counter cation (32000 ± 5000 % M^{-1}) to the slopes of the lines where the counter cations are Base–H^+ (95000 ± 8000 % M^{-1}; Figure AD.5) and Proton Sponge™–H^+ (5000 ± 1000 % M^{-1}; Figure AD.S9 in the Supporting Information) reveals that Na^+ facilitates Co–C heterolysis. It is, however, 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 AD.5 is that *all* the Co–C heterolysis above the intercept of 5 ± 4% and falling on or below this line *appears to be due to the [Base-H^+][$HOCH_2CH_2O^-$]-assisted pathway*. This is an important, previously unappreciated finding.

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 concluded,[23] as the key player in causing increased Co–C heterolyis with such added bases. 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 in the very valuable contribution of others,[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 added, important 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, non-modeled 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 why stable [AdoCbi•(Bulky Base)]$^+$ 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 report that the cobaloxime complexes bind 2-NH_2-py or 1,2-Me_2-Im are interesting results of interest to inorganic chemists in general. *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 "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 need to use only cobamides as B_{12} models wherever possible, or to interpret with extreme caution other "B_{12} model" data as it *might* apply to coenzyme B_{12}•Enzymes themselves, is again emphasized. The (vi) need for great care in interpreting rate laws is another, albeit already well known, take-home message emphasized by the present work. We erred when we concluded 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". The more precise conclusion should have been that our kinetics required " that the bulky ligand is involved *prior to or in* the rate-determining step". Although we believed at the time that our controls ruling out impurities, or the glycolate anion hypothesis, allowed us to make the first statement, the present work show that, in hindsight, the second, more cautious conclusion above is better.

Final take-home message include: (vii) one main basis for the "transition-state mechanochemical triggering" hypothesis for the acceleration of Co–C homolysis discussed elsewhere[13,36] would seem to be hereby taken away (i.e., no evidence for a [AdoCbi•Bulky Base]‡ species exists at present); (viii) the need for open, clear communication in science is reemphasized,[87] especially when puzzling or experimentally conflicting results or interpretations are obtained; and (ix) *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.

What we find most heartening from this study is that that the apparently correct answer resulted in a relatively short period of time, *despite* the five misleading pieces of data or artifacts cited earlier which proved impossible to navigate correctly earlier.[1,23] The most important take-home message, then, is that a proper scientific method, involving attempted disproof of all conceivable alternative hypotheses as Platt has admonished, is indeed, powerful ("For exploring the unknown, there is no faster method").[63]

Experimental

Materials

Each of the following was used as received: adenosylcobalamin (AdoCbl; Sigma, 98%), argon (General Air), ethylene glycol (Aldrich, 99.8% anhydrous), $Ce(NO_3)_3 \bullet 6\ H_2O$ (Aldrich, 99.99% fresh bottle), sodium hydroxide (Fisher Scientific, A.C.S. grade), ammonium hydroxide (Mallinckrodt AR-A.C.S. grade, 29.3%), methanol (Fisher Scientific, HPLC grade), sodium chloride (Fisher Scientific, A.C.S. grade), silver nitrate (Aldrich A.C. S. reagent grade), phosphoric acid (Mallinckrodt AR-A.C.S. grade), potassium phosphate dibasic (Mallinckrodt AR-A.C.S. grade) sodium tetrafluoroborate (Aldrich), reagent alcohol (Fisher Scientific; anhydrous, ~90 % ethanol, ~5% methanol, ~5% isopropyl alcohol), sodium acetate (Mallinckrodt AR-A.C.S. grade), acetic acid (Mallinckrodt AR-A.C.S. 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 nano-pure filtration system. The affinity distillation reagent, $Co(C_2(DO)(DOH))Br_2$, was synthesized by literature methods[88] with > 90% purity as judged by ^1H 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 Materials for the interested reader. The product was characterized by UV-visible spectroscopy, HPLC, and ^1H 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 in / 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 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 hrs.

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: 5.0 mL of 2-Me-py 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 minutes in the first distillation cycle, analogous to the color change from green to red observed by Marzilli and co-workers 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.

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, then putting the vial in a –4 °C freezer for ~2 hours. 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 hrs. 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 reported melting point, 37-39 °C).

The non-sterically 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 non-spinning mode for sufficient N-Me-Im to be collected.

Instrumentation and Equipment

UV-visible absorption spectra (± 1 nm) were recorded on a Hewlett-Packard Model 8452A UV-visible 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 x 4.6 mm Alltech C-18 reverse phase column. ^1H 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 x 10^{-3} mol) of AdoCbi$^+BF_4^-$ was weighed into a foil wrapped vial and taken into the drybox. Inside the drybox, ~31.2 mg (~2 x 10^{-4} mol) of solid TEMPO radical trap were 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 x 10^{-4} M AdoCbi$^+BF_4^-$ (and ~ 2 x 10^{-2} M TEMPO) solution. Next, 1.5 mL aliquots of this solution was transferred into a 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 x 10^{-3} M AdoCbi$^+BF_4^-$ and the appropriate concentration (0.15 to 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-visible spectrum of each cell was followed by periodically removing it from the oil bath, taking a UV-visible spectrum, 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 hrs for the added N-Me-Im or 1,2-Me_2-Im, and ≥ 156 hrs 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 section) 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] x 100%,[5] or by [adenine] / [initial Co(II)Cbi^{+}] x 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 hrs was used.

Co(II)Cobinamide Titration With Axial Bases

Co(II)Cbi^{+}BF$_4^{-}$ titrations with axial bases were performed in a similar manner 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 of a drybox, sealed in a Schlenk cuvette and placed 30 cm in front of a General Electric 275 watt "Sun Lamp" for 20 hrs. The UV-visible 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-visible spectra was taken. This process was repeated until the solutions were ~ 2 M in base concentration.

The titration results are available in the Supporting Materials, with purified N-Me-Im (Figure AD.S1); purified and commercial 1,2-Me_2-Im (Figure AD.S1 and Figure AD.S3), purified pyridine (Figure AD.S4); purified and commercial 2,6-Me_2-py (Figure AD.S5 and AD.S6); and commercial 2-Me-py (Figure AD.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 (~20000 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[1] 5 and[3] *5'* are hereby replaced by the correct Figure AD.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 for erroneous $Co(II)Cbi^+BF_4^-$ titration results)[3] were analyzed by GC-MS (see instrumentation and equipment section). A 10 uL 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,[89] indicating that any possible impurity either : (i) was not present in quantities above our detection limit ($\geq$ 0.5 %); (ii) was not obtained by the sampling method employed; (iii) has the same retention time as the base being tested under the conditions employed; (iv)

was retained by the GC column; or (v) was not detectable by the MS detector. In any case, the method did not prove useful and was not pursued further.

Supporting Information

Supporting Information is available as usual from the ACS web site as well as in the Ph. D. dissertation of K. M. Doll (Colorado State University Spring 2003): Table AD.S1, A comparison of the percent heterolysis of $AdoCbi^+$ thermolyses with various purities of added imidazoles; Table AD.S2, A comparison of the percent heterolysis of $AdoCbi^+$ thermolyses with various purities of added sterically hindered pyridines; Table AD.S3, A comparison of the percent heterolysis and rate of AdoCbl thermolyses with various purities of imidazoles; Figure AD.S1-ADS.7, Titrations of $Co(II)Cbi^+BF_4^-$ with commercial or purified bases; Figure AD.S8, A plot of % heterolysis of the Co–C bond vs the calculated concentration of [Proton Sponge™-H^+glycolate$^-$]; Figure AD.S9, A plot of % heterolysis of the Co–C bond vs the concentration of added [Na^+Glycolate$^-$]; Section AD.S-1, The derivation of the kinetic rate law for Scheme AD.1; Section AD.S-2, Adenosylcobinamide Synthesis Procedure. This material is available free of charge via the Internet at http://pubs.acs.org.

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(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; (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, 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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(66) Aoki, M.; Hara, Y. In *Jpn. Kokai Tokkyo Koho*; (Tosoh Corp., Japan).: JP, 2000; p 5 pp.
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(81) The pKa of the heterolysis penultimate product Adeninyl-, should lie above the pKa = 9.8 of Adenine.[82,83]
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(87) Unfortunately, there was a breakdown of the refereeing and other communication channels in the present case. Had the PI been afforded the courtesy of seeing a preprint either from the journal or the authors of reference,[23] then the noted lapses in logic in that paper[23] would have been pointed out prior to publication of their paper. Surely that would have led to at least a smoother if not a more insightful scientific process, one whose convergence on the "insidious impurity problem"[52] addressed here would have been more efficient on journal space, reader time, and individual effort of the two groups involved. A more cooperative mode of science is the more efficient, modern way to do science in our opinion.
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Supporting Information

Adenosylcobinamide Plus Exogenous, Sterically Hindered, Putative Axial-Bases: A Re-investigation Into the Cause of Record Levels of Co–C Heterolysis

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Table AD.S1

A comparison of the percent heterolysis of the Co–C bond of AdoCbi $^{+}$ with various purities of added imidazoles.

Base	% Heterolysis
N-Me-Im Data	
N-Me-Im (> 2 Years Old)	41 ± 7
N-Me-Im (Fresh Bottle)	56 ± 7
N-Me-Im (Fresh Bottle)	64 ± 7
N-Me-Im (Fresh Bottle)	47 ± 7
1,2-Me_2-Im Data	
1,2-Me_2-Im (> 2 Years Old)	84 ± 7
1,2-Me_2-Im (> 2 Years Old)	86 ± 7
1,2-Me_2-Im (Fresh Bottle)	81 ± 7
1,2-Me_2-Im (Recrystallized)	90 ± 7
1,2-Me_2-Im (Recrystallized)	72 ± 7

Kinetic Product Studies of the Thermolyses with Pyridines

The HPLC study of products from $AdoCbi^+$ with added sterically hindered pyridines were performed on nine different reaction solutions (Table AD.S2). As in the imidazole case, the purity of the axial base, including bases purified by affinity distillation, did not show any trend towards more, or less, Co–C heterolysis.

The percent heterolysis for all of the pyridines is the same within experimental error at 16 ± 5 %. These results are intermediate between of the earlier report (24 % for 2,6-Me_2-py, and 6 % for 2-Me-py),[1] leading to the conclusion that all of the pyridines give approximately the same enhancement of heterolysis in the $AdoCbi^+$ Co–C bond cleavage. The fact that all of the pyridine reaction systems display the same percent heterolysis despite different Co–N bond lengths[2,3] shows that binding of the axial base is not involved in Co–C heterolysis. The rate constant for the thermolysis reaction (1.1 (± 0.1) x 10^{-5} s^{-1}) is ~ 3 times faster than the base-free reaction, but ~ 3 times slower than the reaction with added imidazoles, data consistent with our previous report.[1]

Table AD.S2

A comparison of the percent heterolysis of the Co–C bond of $AdoCbi^+$ with various purities of added sterically hindered pyridines.

Base	% Heterolysis
2-Me-py Data	
2-Me-py (Fresh Bottle)	17 ± 7
2-Me-py (Fresh Bottle)	16 ± 7
2-Me-py (Fresh Bottle)	25 ± 7
2-Me-py (Spinning-Band Distilled)	26 ± 7
2-Me-py (Spinning-Band Distilled)	15 ± 7
2-Me-py (Affinity Distilled)	21 ± 7
2-Me-py (Affinity Distilled)	13 ± 7
2,6-Me_2-py Data	
2,6-Me_2-py (> 2 years old)	15 ± 7
2,6-Me_2-py (Fresh Bottle)	19 ± 7
2,6-Me_2-py (> 2 Years Old)	16 ± 7
2,6-Me_2-py (> 2 Years Old)	19 ± 7
2,6-Me_2-py (Fresh Bottle)	26 ± 7
2,6-Me_2-py (Fresh Bottle)	23 ± 7
2,6-Me_2-py (Spinning-Band Distilled)	9 ± 7

Table AD.S3

A comparison of the percent heterolysis of the Co–C bond of AdoCbl with various purities of imidazoles.

Base	k_{obs} (s^{-1})	% Heterolysis
N-Me-Im Data		
N-Me-Im > 2 years old	$1.7 \pm 0.2 \times 10^{-4}$	5 ± 5
N-Me-Im Fresh Bottle	$2.0 \pm 0.2 \times 10^{-4}$	6 ± 5
1,2-Me_2-Im Data		
1,2-Me_2-Im > 2 years old	$1.7 \pm 0.2 \times 10^{-4}$	8 ± 5
1,2-Me_2-Im Recrystallized	$2.1 \pm 0.2 \times 10^{-4}$	7 ± 5
None	$1.7 \pm 0.2 \times 10^{-4}$	2 ± 5

Titration of Co(II)Cbi$^+$ with imidazoles

Because they have not been previously reported, we titrated Co(II)Cbi$^+$BF$_4^-$ with N-Me-Im and 1,2-Me$_2$-Im (Figures AD.S1, AD.S2, and AD.S3). The results are those now expected.[4] There is binding by the non-hindered N-Me-Im, but no detectable binding by the sterically hindered 1,2-Me$_2$-Im.[1]

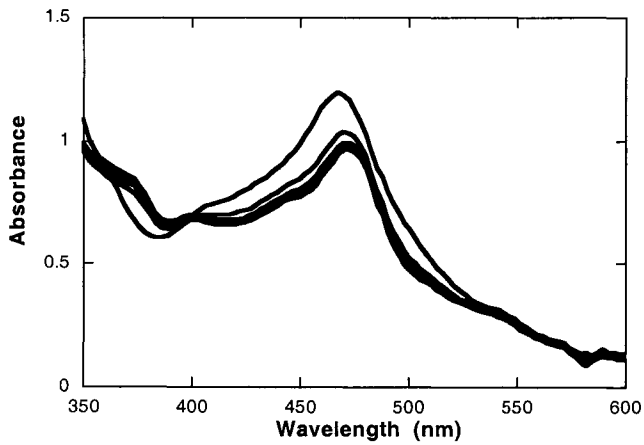

Figure AD.S1 A UV-visible spectroscopy titration of Co(II)Cbi$^+$BF$_4^-$ (~ 1 x 10^{-4} M) with spinning-band-column distilled N-Me-Im in ethylene glycol solution. The concentrations of N-Me-Im are 0, 0.12, 0.25, 0.48, 0.93, 1.72, and 2.09 M. The results show a reaction similar to that of pyridine, the other unhindered base studied herein.

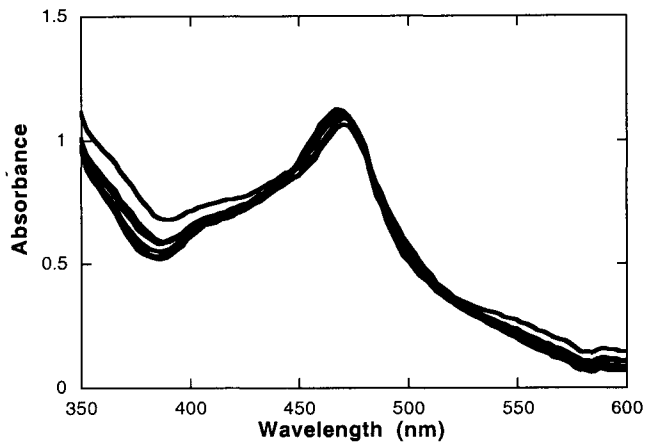

Figure AD.S2 A UV-visible spectroscopy titration of Co(II)Cbi^{+}BF$_4^{-}$ (~ 1 x 10^{-4} M) with a solution of 1,2-Me$_2$-Im (recrystallized twice; 3.7 M in ethylene glycol) in ethylene glycol solution. The concentrations of 1,2-Me$_2$-Im are 0, 0.07, 0.14, 0.28, 0.62 and 1.07 M. The results show no detectable reaction as observed for other hindered bases studied herein (2-Me-py and 2,6-Me$_2$-py). It is also of note that a similar titration with unpurified 1,2-Me$_2$-Im (Figure AD.S3) also did not show any reaction with Co(II)Cbi^{+}BF$_4^{-}$. (The absorbance increase at higher concentrations is caused by the non-zero absorbance of the high concentrations of base, especially at low wavelengths.)

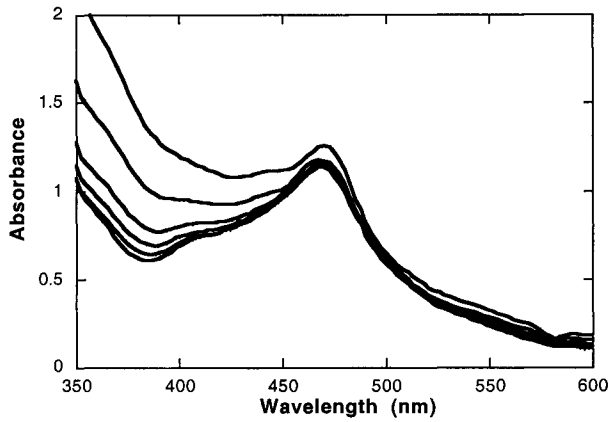

Figure AD.S3 A UV-visible spectroscopy titration of Co(II)Cbi$^{+}BF_4^{-}$ (~ 1 x 10^{-4} M) with a solution (7.5 M) of commercial 1,2-Me_2-Im in ethylene glycol solution. The concentrations of 1,2-Me_2-Im are 0, 0.14, 0.28, 0.53, 1.21, and 2.07 M. The results show no significant reaction, but a large baseline shift caused by the absorbance of the dark-brown unpurified base.

Titration of Co(II)Cbi$^+$ with pyridines

The titrations of Co(II)Cbi$^+$BF$_4^-$ with pyridine and the sterically hindered pyridines, 2-Me-py and 2,6-Me$_2$-py, showed results similar to those in Figure 7 of a report in the literature.[4] It can be concluded that Co(II)Cbi$^+$BF$_4^-$ binds pyridine (Figure AD.S4) but does not bind sterically hindered pyridines such as 2,6-Me$_2$-py to an appreciable extent (Figure AD.S5). The earlier reports of binding of 2,6-Me$_2$-py have proven to be irreproducible.[1,5] Figures 5 and 5' from the earlier report and correction,[1,5] should be replaced with Figure AD.S5 herein. It is of note that even commercial 2,6-Me$_2$-py and 2-Me-py do not show binding by UV-visible spectroscopy; instead, they show only the expected baseline increase at lower wavelengths (Figures AD.S6 and AD.S7 in the Supporting Information); the commercial bases are visibly orange or yellow, which makes their absorbance non-zero at the high concentrations studied.

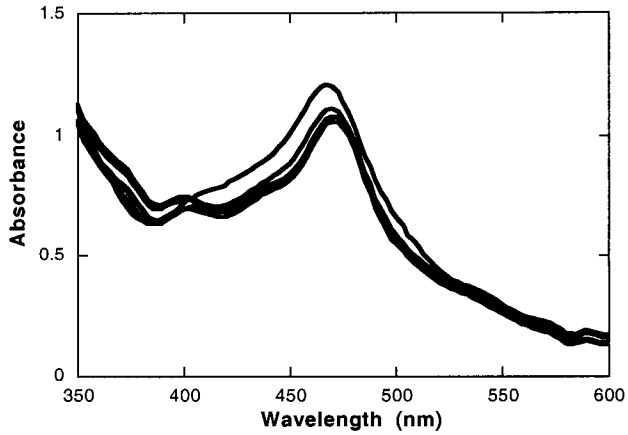

Figure AD.S4 A UV-visible spectroscopy titration of Co(II)Cbi$^{+}BF_4^{-}$ (~ 1 x 10^{-4} M) with spinning-band-column distilled pyridine in ethylene glycol solution. The concentrations of pyridine are 0, 0.05, 0.12, 0.24, 0.47, 0.92, 1.71, and 2.85 M. The results show a detectable reaction consistent with literature reports.[1,4,5]

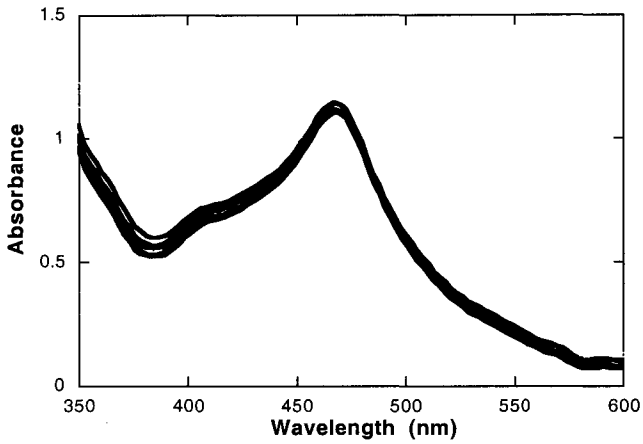

Figure AD.S5 A UV-visible spectroscopy titration of Co(II)Cbi$^{+}BF_4^{-}$ (~ 1 x 10^{-4} M) with spinning-band-column distilled 2,6-Me_2-py in ethylene glycol solution. The concentrations of 2,6-Me_2-py are 0, 0.17, 0.33, 0.64, 1.18 and 1.66 M. The results show no significant reaction, consistent with the literature.[4] The slight absorbance change at lower wavelength is due to the non-zero absorbance of the base (the solution volume is ~20% 2,6-Me-py for the final spectrum).[6] This figure should replace the erroneous Figures 5 and 5' in earlier reports.[1,5]

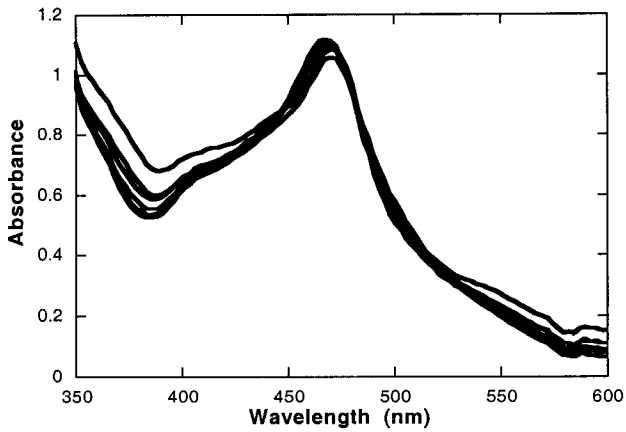

Figure AD.S6 A titration UV-visible spectroscopy of Co(II)Cbi+BF4- (~ 1 x 10-4 M) with commercial 2,6-Me2-py in ethylene glycol solution. The concentrations of 2,6-Me2-py are 0, 0.15, 0.28, 0.54, 1.01, and 1.81 M. The results show no significant reaction. The small baseline shift caused by the absorbance of the slightly yellow-unpurified base.

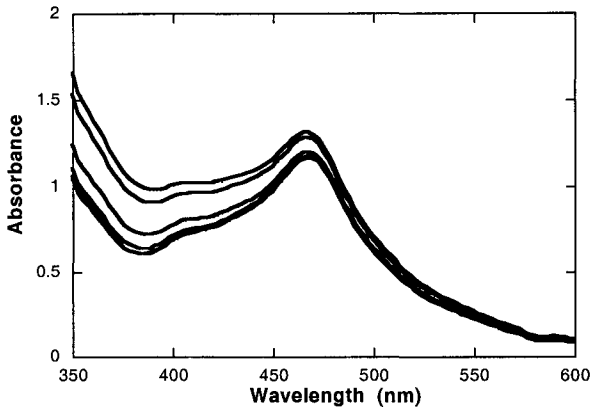

Figure AD.S7 A UV-visible spectroscopy titration of Co(II)Cbi+BF4- (~ 1 x 10-4 M) with commercial 2-Me-py in ethylene glycol solution. The concentrations of 2-Me-py are 0, 0.17, 0.32, 0.63, 1.26, and 1.45 M. The results show no significant reaction. The large baseline shift caused by the absorbance of the dark-yellow / orange unpurified base.

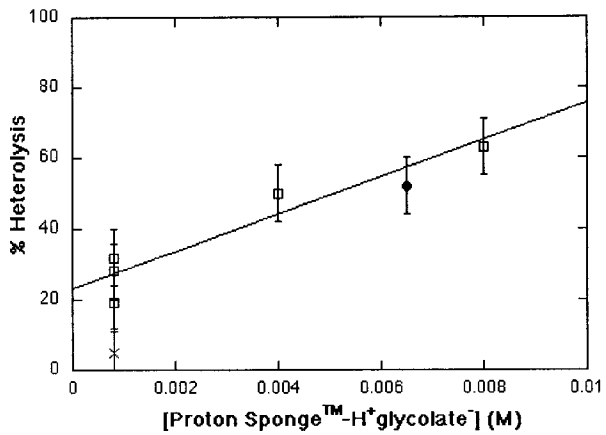

Figure AD.S8 A plot of % heterolysis of the Co–C bond vs the calculated concentration of [Proton Sponge™-H+glycolate-]. The slope and intercept of the line are 5000 (1000 % M-1 and 23 (4 % respectively. The results from this work (() show a linear correlation; the data also correlates well with the result reported in 1996 ADDIN ENRfu 7 (), but not with that reported in 1999 (). ADDIN ENRfu 1 We suspect the 1999 point (() is in error due to the failure ADDIN ENRfu 8 to fully dissolve the kinetically insoluble Proton Sponge™. The lower % heterolysis in this system, compared to the 83 ± 7 % observed in the 1,2-Me_2-Im system at a calculated 0.0008 M glycolate anion concentration, can be explained by the differences in the cations, Proton Sponge™-H^+ (pKa, 12.3) vs 1,2-Me_2-Im-H^+ (pKa 7.8), involved in the Co–C heterolysis reactions, as discussed in the main text.

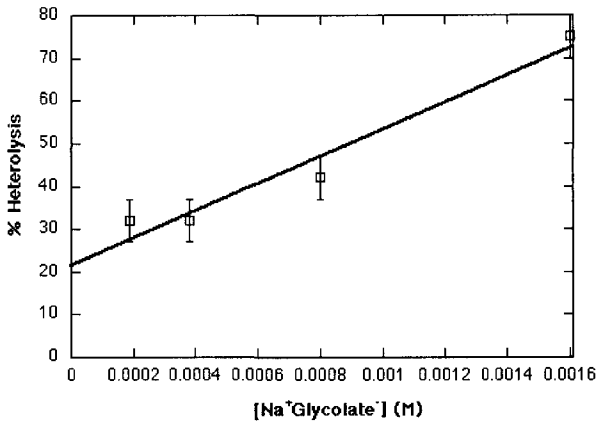

Figure AD.S9 A plot of % heterolysis of the Co–C bond vs the concentration of added [Na^+glycolate$^-$]. The slope and intercept of the line are 32000 ± 5000 % M^{-1} and 22 ± 4 % respectively. The lower % heterolysis in this system, compared to the 83 ± 7 % observed in the 1,2-Me_2-Im system at a calculated 0.0008 M glycolate anion, can be explained by the differences in the cations, Na^+ vs 1,2-Me_2-Im-H^+, involved in the Co–C heterolysis reactions, as discussed in the main text.

Section AD.S-1

The derivation of the kinetic rate law for Scheme AD.1

The rate law for Scheme AD.1 is analogous a similar to the derivation in the Supporting Information elsewhere.[1,7]

Key definitions and abbreviations:

$[AdoCbi^+] = [AdoCbi^+ \bullet solvent]$

T = total

$[Base\text{-}H^+Glycolate^-] = [BH^+Gly^-]$

[Product Ratio] = [Heterolysis products] / [Homolysis products]

= [Adenine]/[Ado$^\bullet$-derived products]

Mass balance equations

$[AdoCbi^+]_T = [AdoCbi^+] + [AdoCbi^+ \bullet Base]$

$[Base]_T = [Base] + [AdoCbi^+ \bullet Base]$

Under our conditions, $[Base] >> [AdoCbi^+ \bullet Base]$, so

$[Base]_T = [Base]$

$$(1a) \quad K_{assoc.} = \frac{[AdoCbi^+ \bullet Base]}{[AdoCbi^+][Base]}$$

$$(1b) \quad [AdoCbi^+] = \frac{[AdoCbi^+ \bullet Base]}{K_{assoc.}[Base]}$$

Using equation 1a and the mass balance equation for $[AdoCbi^+]_T$ gives:

$$(2a)\quad \left[AdoCbi^+\right]_T = \frac{\left[AdoCbi^+ \bullet Base\right]}{K_{assoc.}[Base]} + \left[AdoCbi^+ \bullet Base\right]$$

$$(2b)\quad \left[AdoCbi^+\right]_T = \left[\frac{\left[1 + K_{assoc.}[Base]\right]}{K_{assoc.}[Base]}\right]\left[AdoCbi^+ \bullet Base\right]$$

$$(2c)\quad \left[AdoCbi^+ \bullet Base\right] = \left[AdoCbi^+\right]_T\left[\frac{K_{assoc.}[Base]}{\left[1 + K_{assoc.}[Base]\right]}\right]$$

The appropriate kinetic equations for Scheme AD.1

$$(3)\quad \frac{d\left[Co^{II}Cbi^+\right]_T}{dt} = \frac{-d\left[AdoCbi^+\right]}{dt} = k_{off,hom}\left[AdoCbi^+\right] + k_{off,het}\left[AdoCbi^+\right]\left[BH^+Gly^-\right] + k_{on,hom}\left[AdoCbi^+ \bullet Base\right] + k_{on,het}\left[AdoCbi^+ \bullet Base\right]\left[BH^+Gly^-\right]$$

Substitution for [AdoCbi$^+$] from equation 1b into equation 3 gives:

$$(4a)\quad \frac{-d\left[AdoCbi^+\right]}{dt} = k_{off,hom}\frac{\left[AdoCbi^+ \bullet Base\right]}{K_{assoc.}[Base]} + k_{off,het}\frac{\left[AdoCbi^+ \bullet Base\right]}{K_{assoc.}[Base]}\left[BH^+Gly^-\right] + k_{on,hom}\left[AdoCbi^+ \bullet Base\right] + k_{on,het}\left[AdoCbi^+ \bullet Base\right]\left[BH^+Gly^-\right]$$

$$(4b)\quad \frac{-d\left[AdoCbi^+\right]}{dt} = \left[AdoCbi^+ \bullet Base\right] \times \left[\frac{k_{off,hom}}{K_{assoc.}[Base]} + \frac{k_{off,het}}{K_{assoc.}[Base]}\left[BH^+Gly^-\right] + k_{on,hom} + k_{on,het}\left[BH^+Gly^-\right]\right]$$

Substitution for [AdoCbi$^+$•Base] from equation 2c into equation 4b gives:

$$(5a)\quad \frac{-d[AdoCbi^+]}{dt} = [AdoCbi^+]_T \left[\frac{K_{assoc.}[Base]}{[1+K_{assoc.}[Base]]}\right] \times \left[\frac{k_{off,hom}}{K_{assoc.}[Base]} + \frac{k_{off,het}}{K_{assoc.}[Base]}[BH^+Gly^-] + k_{on,hom} + k_{on,het}[BH^+Gly^-]\right]$$

$$(5b)\quad \frac{-d[AdoCbi^+]}{dt} = [AdoCbi^+]_T \times \left[\frac{k_{off,hom} + k_{off,het}[BH^+Gly^-] + [K_{assoc.}[Base][k_{on,hom} + k_{on,het}[BH^+Gly^-]]]}{[1+K_{assoc.}[Base]]}\right]$$

A first order plot of equation 5b yields equation 6:

$$(6)\quad k_{obs} = \left[\frac{k_{off,hom} + k_{off,het}[BH^+Gly^-] + [K_{assoc.}[Base][k_{on,hom} + k_{on,het}[BH^+Gly^-]]]}{[1+K_{assoc.}[Base]]}\right]$$

Under condition where $1 >> K_{assoc}[Base]$ and $k_{on,het}$ is small, k_{obs} is given be equation 7. This is similar to the expression 6C in the Supporting Information elsewhere.[1,7]

$$(7)\quad k_{obs} = k_{off,hom} + k_{off,het}[BH^+Gly^-] + k_{on,hom}K_{assoc.}[Base]$$

We can also derive the product ratio analogously to that derived in the Supporting Information elsewhere.[1,7]

From Scheme AD.1

$$(8)\quad \frac{d[\text{Adenine}]}{dt} = k_{off,het}\left[BH^{+}Gly^{-}\right]\left[AdoCbi^{+}\right] + k_{on,het}\left[AdoCbi^{+}\bullet Base\right]\left[BH^{+}Gly^{-}\right]$$

Substitution for [AdoCbi^{+}•Base] from equation 1 into equation 8 gives:

$$(9a)\quad \frac{d[\text{Adenine}]}{dt} = k_{off,het}\left[BH^{+}Gly^{-}\right]\left[AdoCbi^{+}\right] + k_{on,het}K_{assoc.}[Base]\left[AdoCbi^{+}\right]\left[BH^{+}Gly^{-}\right]$$

$$(9b)\quad \frac{d[\text{Adenine}]}{dt} = \left[AdoCbi^{+}\right]\left[k_{off,het}\left[BH^{+}Gly^{-}\right] + k_{on,het}K_{assoc.}[Base]\left[BH^{+}Gly^{-}\right]\right]$$

From Scheme AD.1

$$(10)\quad \frac{d[\text{Ado}^{\bullet}\text{products}]}{dt} = k_{off,hom}\left[AdoCbi^{+}\right] + k_{on,hom}\left[AdoCbi^{+}\bullet Base\right]$$

Substitution for [AdoCbi^{+}•Base] from equation 1 into equation 10 gives:

(11a) $$\frac{d[Ado^{\bullet}products]}{dt} = k_{off,hom}\left[AdoCbi^{+}\right] + k_{on,hom}K_{assoc.}[Base]\left[AdoCbi^{+}\right]$$

(11b) $$\frac{d[Ado^{\bullet}products]}{dt} = \left[AdoCbi^{+}\right]\left[k_{off,hom} + k_{on,hom}K_{assoc.}[Base]\right]$$

Taking the ratio, equation 9b / equation 11b gives:

(12) $$\frac{\frac{d[Adenine]}{dt}}{\frac{d[Ado^{\bullet}products]}{dt}} = \frac{\left[AdoCbi^{+}\right]\left[k_{off,het}\left[BH^{+}Gly^{-}\right] + k_{on,het}K_{assoc.}[Base]\left[BH^{+}Gly^{-}\right]\right]}{\left[AdoCbi^{+}\right]\left[k_{off,hom} + k_{on,hom}K_{assoc.}[Base]\right]}$$

(13) $$\frac{\int_{0}^{[Adenine]_t} d[Adenine]}{\int_{0}^{[Ado^{\bullet}products]_t} d[Ado^{\bullet}products]} = \frac{\left[k_{off,het}\left[BH^{+}Gly^{-}\right] + k_{on,het}K_{assoc.}[Base]\left[BH^{+}Gly^{-}\right]\right] \times \int_{t=0}^{t=t}\left[AdoCbi^{+}\right]dt}{\left[k_{off,hom} + k_{on,hom}K_{assoc.}[Base]\right] \times \int_{t=0}^{t=t}\left[AdoCbi^{+}\right]dt}$$

Cancellation of identical terms and integration of equation 13 gives:

(14) $$\frac{[Adenine]}{[Ado^{\bullet}products]} = \frac{k_{off,het}\left[BH^{+}Gly^{-}\right] + k_{on,het}K_{assoc.}[Base]\left[BH^{+}Gly^{-}\right]}{k_{off,hom} + k_{on,hom}K_{assoc.}[Base]}$$

This expression is similar to expression 11b in the Supporting Information elsewhere.[7]

If the base must be dissociated to allow an ethylene glycolate anion effect, then $k_{on,het} << k_{off,het}$ and equation 14 simplifies.

(15) $$\frac{[Adenine]}{[Ado^{\bullet}products]} = \frac{k_{off,het}\left[BH^{+}Gly^{-}\right]}{k_{off,hom} + k_{on,hom}K_{assoc.}[Base]}$$

Section AD.S-2 Adenosylcobinamide Synthesis Procedure

The compound AdoCbi$^{+}BF_4^{-}$ was synthesized from AdoCbl using $Ce(OH)_3$ according to the procedure of Hay.[9] This is a modified version of a procedure originally reported by Freidrich and Bernhauer.[10,11] Following the procedure of Renz,[12] $Ce(OH)_3$ was generated from $Ce(NO_3)_3 \bullet 6\ H_2O$ with a 10% NaOH solution. Desalting, purification, and ion exchange were accomplished using column chromatography as described below.

A $Ce(OH)_3$ slurry was prepared on the by dissolving 13.7 g (31.6 mmol) of $Ce(NO_3)_3 \bullet 6\ H_2O$ was in 100 mL of H_2O in a 250 mL plastic centrifuge bottle. Next, 50 mL of 10% NaOH (~10% by mass; 5.05 g NaOH / 50 mL H_2O) was added dropwise with rapid stirring. The resulting suspension was spun in a centrifuge (5 min at 2000 RPM) and the solution was decanted off. Then, 50 mL of 0.3% ammonia solution (0.3% by volume; 4.09 mL of concentrated 29.3% solution diluted to 400 mL with H_2O) was added and the centrifugation was repeated. This ammonia washing was performed a total of 4 times; the suspension turned purple after the second ammonia washing.

Under red light conditions, in a 500 mL 3-neck flask, 350 mL of H_2O was heated to 77 °C in an oil bath and 506 mg (0.32 mmol) of AdoCbl was added. The freshly prepared $Ce(OH)_3$ slurry was suspended in 50 mL of H_2O and slowly added to the AdoCbl solution with rapid stirring and with careful monitoring of the temperature and the pH, the later using a pH electrode. The reaction was allowed to stir for 75 min, with the temperature maintained between 75 and 80 °C and the pH remaining between 8-10.

The product containing solution was decanted and saved. The remaining slurry was washed 3 times with 0.3% NH_4OH solution, centrifuged, and the washings were

combined with the light-brown product solution. The product solution was filtered through a medium glass frit and the volume of the solution was reduced to 25 mL by rotary evaporation (< 10 torr , < 35 °C for ~6 hrs) resulting in a dark-brown solution.

The solution was desalted on an Amberlite XAD-2 column (3.5 cm x 36.5 cm; column preparation is described below). The product solution was slowly loaded onto the column along with 58 mg of NaCl (the NaCl is not necessary, but helps identify when the salts have completely eluted with the Ag^+ test below). The product was allowed to sit on the column for 10 min before elution with H_2O at 12 mL / min. Fractions coming off the column were checked for Cl^- with 0.2 M $AgNO_3$ solution. The column was eluted with H_2O for 2 hrs, although most of the Cl^- came off of the column in < 10 min. The product was removed from the column by elution with 600 mL of 80% reagent alcohol/20% H_2O at ~ 5 mL / min for 2 hrs. The volume of this solution was reduced to ~ 50 mL by rotary evaporation (< 10 torr , < 35 °C for ~4 hrs).

Unreacted starting material and other cobalamin and cobinamide impurities were removed by loading the solution onto an SP Sephadex C25 column (3.5 cm x 41 cm). The column was eluted with H_2O until the fractions coming off of the column were colorless as judged by UV-visible absorbance < 0.2 (~10 hrs at ~5 mL / min). The product was removed from the column by elution with 0.01 M potassium phosphate buffer, pH 7.0 (~ 5 mL / min, ~ 7 hrs). Fractions were monitored for product by UV-visible spectroscopy, combined, and their volume reduced by rotary evaporation to 20 mL (< 10 torr , < 35 °C for ~8 hrs).

Another Amberlite XAD-2 desalting column was used to remove phosphate, using the same procedure as stated above. This solution was loaded onto a DEAE

cellulose column (1 cm x 8 cm) and eluted with water to exchange the OH^- anion to the BF_4^- anion.

The product was characterized by UV-visible spectroscopy, HPLC, and ^1H 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) monitored at both 254nm and 302 nm, and ~ 90% by ^1H NMR of the aromatic region which has been shown to be a useful criteria of purity in cobalamins.[13] The overall yield was 116.8 mg (37 %). The literature yield of the OH^- using a phenol extraction step instead of the desalting column employed above is 50 %.[9]

Preparation of Columns

All columns were prepared according to manufacturers instructions: Amberlite XAD-2 (Supelco) resin (~3 g of resin will generate a 3.5 cm x 1 cm column) was stirred in MeOH, allowed to settle for 15 min, then decanted to remove fines. This process was repeated 4 times using H_2O as the solution with the 4th resin suspension being poured into the column and allowed to settle. SP Sephadex C25 (Sigma) resin was soaked in H_2O for 5 hrs and then packed into a column. It was charged by running 2 L of 0.5 M NaCl solution through the column, then elution with ~1 L of pure H_2O until no Cl^- could be detected with 0.2 M $AgNO_3$ solution. DEAE cellulose (Sigma) was suspended in H_2O, allowed to settle, and decanted 3 times, then suspended in a 0.1 M NaOH, 0.5 M NaCl solution for 5 min. It was then washed with H_2O, and subsequently suspended in 0.1 M HCl for 10 min. Next, it was repeatedly washed until the pH was 5.1, then suspended in 1 M NaCl for 10 min. Finally, it was washed with H_2O and suspended and stored in $NaBF_4$ at 4 °C until needed. After pouring the resin into the column, it was washed with 20 mL of H_2O before the addition of the reaction mixture.

References and Notes

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