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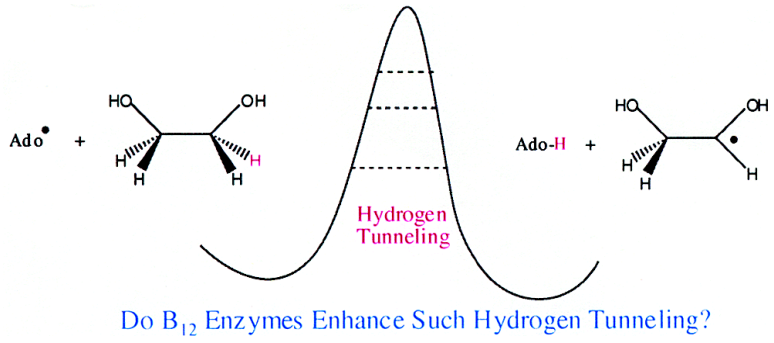

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The First Experimental Test of the Hypothesis that Enzymes Have Evolved To Enhance Hydrogen Tunneling

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Abstract: The literature 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 hydrogen 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)• atom abstraction from ethylene glycol-d_0 and ethylene glycol-d_4 solvent by 5′-deoxyadenosyl radical (Ado•) and (ii) the same H• abstraction reactions by the 8-methoxy-5′-deoxyadenosyl radical (8-MeOAdo•). The Ado• and 8-MeOAdo• radicals are generated by Co−C thermolysis of their respective precursors, AdoCbl and 8-MeOAdoCbl. Deuterium kinetic isotope effects (KIEs) of the H•(D•) abstraction reactions from ethylene glycol have been measured over a temperature range of 80−120 °C: KIE = 12.4 ± 1.1 at 80 °C for Ado• and KIE = 12.5 ± 0.9 at 80 °C for 8-MeOAdo• (values ca. 2-fold that of the predicted maximum primary times secondary ground-state zero-point energy (GS-ZPE) KIE of 6.4 at 80 °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• and 2.1 ± 0.6 kcal mol^{-1} for 8-MeOAdo• have been obtained, both of which are significantly larger than the nontunneling, zero-point energy only maximum of 1.2 kcal mol^{-1}. Pre-exponential factor ratios (A_H/A_D) of 0.16 ± 0.07 for Ado• and 0.5 ± 0.4 for 8-MeOAdo• are observed, both of which are significantly less than the 0.7 minimum for nontunneling behavior. The data provide strong evidence for the expected quantum mechanical tunneling in the Ado• and 8-MeOAdo•-mediated H• 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•-mediated H• 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 Occam's Razor conclusion is that at least this adenosylcobalamin-dependent enzyme has not evolved to enhance quantum mechanical tunneling, at least within the present error bars. Instead, this B_{12}-dependent enzyme simply exploits the identical level of quantum mechanical tunneling that is available in the enzyme-free, solution-based H• abstraction reaction. The results also require a similar, if not identical, barrier width and height within experimental error for the H• abstraction both within, and outside of, the enzyme.

Introduction

An intriguing[1−4] but controversial[5−7] hypothesis dating back to 1989,[8,9] if not before,[10] is the 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 in, for example, their hydrogen transfer reactions.[10−12] This "enhanced hydrogen tunneling" hypothesis merits careful scrutiny since the availability of many low-frequency motions in proteins[13−20] can change interatomic distances by 0.2 to ca.

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(5) Cui, Q.; Karplus, M. *J. Am. Chem. Soc.* **2002**, *124*, 3093.
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(11) (a) An early version of this hypothesis dates back to a 1971 report by Gold, who studied "facilitated proton transfers" in chymotrypsin and carbonic anhydrase. Gold notes, "...enzymes may act by enforcing small reductions in the length of hydrogen bonds, thereby increasing the probability of quantum mechanical tunneling."[10] (b) Moore and Pearson have noted, "It is correct to say that tunneling is always a factor in reactions involving transfer of hydrogen atoms, protons, or hydride ions." Moore, J. W.; Pearson, R. G. *Kinetics and Mechanism*, 3rd ed.; John Wiley and Sons: New York, 1981; p. 371. The extent of tunneling might, of course, be nearly complete at low temperatures and for penetrable barriers to very little at higher temperatures for barriers that preclude significant tunneling.
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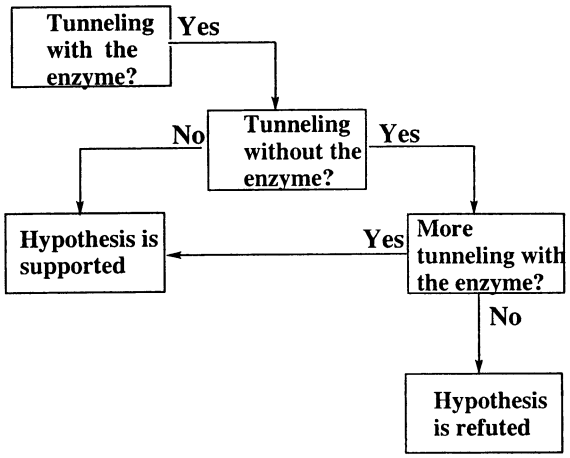
Scheme 1. Simple Three-Step Procedure Testing the Hypothesis That Enzymes Have Evolved To Enhance Quantum Mechanical Tunneling[a]



[a] It is important that the same substrate, or at least a substrate as similar as possible, be used when following this procedure to avoid results that are complicated by substrate-dependent tunneling.

1.5 Å or more,[14] conceivably resulting in enhanced tunneling between thereby closer reaction sites (H• atom-transfer sites, for example, vide infra).[21−29] Since science proceeds through the disproof of alternative hypotheses,[30] ("for exploring the unknown, there is no faster method"),[30] two alternative hypotheses are (1) 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 (2) that the tunneling is actually decreased in the enzyme as a result of a (seemingly unlikely) increase in the reaction barrier width as evolution has decreased 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 1).

However, an experimental test of the enhanced tunneling hypothesis has not appeared until now because of 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 hydrogen tunneling hypothesis. In this enzymic reaction, enzyme-accelerated Co−C homolysis of AdoCbl[35] generates Ado• (at least formally),[36] and this Ado• then abstracts a H• from ethylene glycol substrate ($HOCH_2CH_2OH$-d_0 or its deuterated analogue, $HOCD_2CD_2OH$-d_4). Significantly, this identical reaction is known in solution when enzyme-free AdoCbl is thermolyzed in ethylene glycol solution.[37] Although the needed data probing tunneling for the enzyme diol dehydratase itself does not yet exist, a very similar H• abstraction from a primary C−H bond has been studied recently by Banerjee and coworkers 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), $HOCH_2$-CH_2OH, estimated BDE (C−H) = 91.1−95 kcal mol^{-1} [38,39] vs methylmalonyl-CoA H_3C−R with estimated BDE (C−H) = ∼92−101 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] the Kreevoy criteria being: (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 (8.9 at 20 °C even 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, whereas 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 $1/T$.[42] The value $E_D - E_H$ is obtained from the slope, whereas 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 20 °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 as expected[11b] in the H• abstraction reaction from the H_3C−R group of methylmalonyl-CoA by an incipient Ado•.[43,44] This same system has also been 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

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(26) Basran, J.; Sutcliffe, M. J.; Scrutton, N. S. *J. Biol. Chem.* **2001**, *276*, 24581.
(27) Antoniou, D.; Schwartz, S. D. *J. Phys. Chem. B* **2001**, *105*, 5553.
(28) Bruice, T. C.; Benkovic, S. J. *Biochemistry* **2000**, *39*, 6267.
(29) It has been noted by Bruice and Benkovic, "There is at present, however, little direct evidence as to how enzymes use correlated motion in catalysis. Theoretical treatments have been offered which explicitly recognize the role of protein dynamics in the treatment of hydride transfer reactions and tunneling."[28]
(30) Platt, J. R. *Science* **1964**, *146*, 347.
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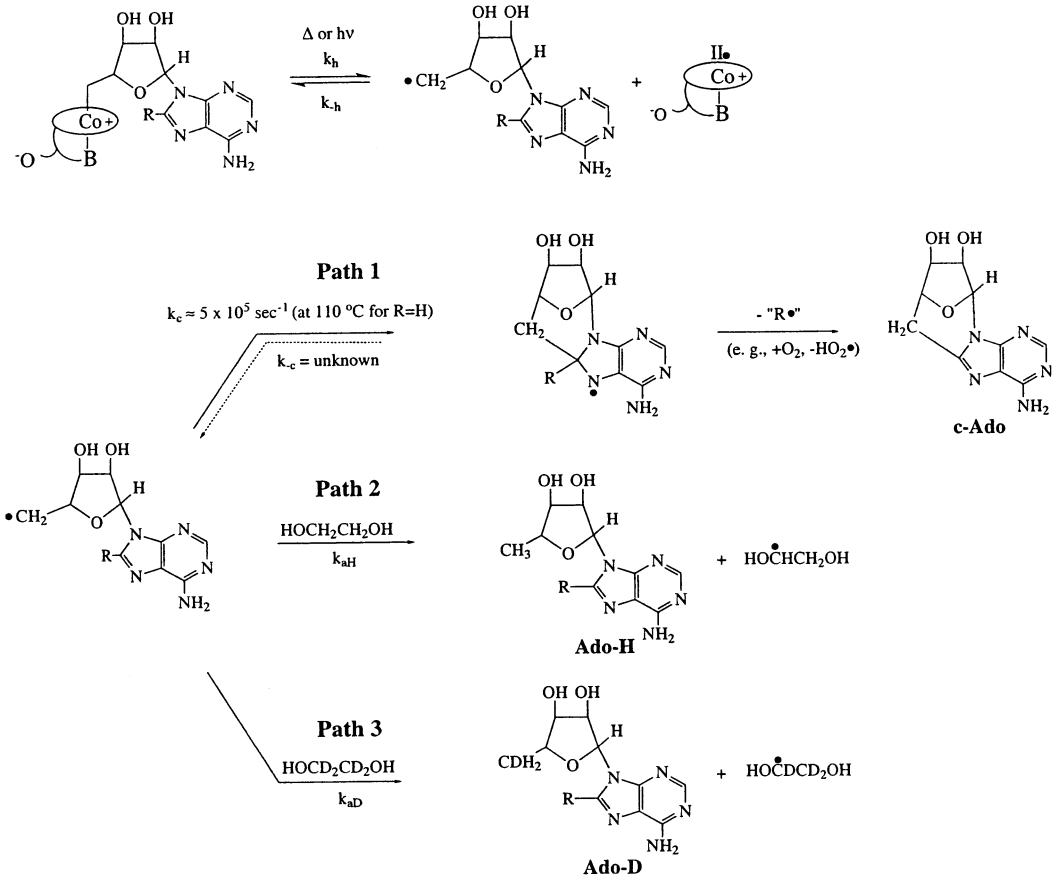
(40) *CRC Handbook of Chemistry and Physics*, 81st ed.; David R. Lide, Ed.; CRC Press: Boca Raton, FL, 2000.
(41) Kwart, H. *Acc. Chem. Res.* **1982**, *15*, 401.
(42) The reason for the ratio of less than one in tunneling cases can be seen by coplotting Arrhenius plots of ln k vs $1/T$ for the hydrogen and deuterium cases. If the tunneling component is large, the rate will be enhanced at low temperatures, causing significant curvature in the hydrogen case. This will make the pre-exponential factor (calculated from the intercept of this line) lower, and the ratio of pre-exponential factors less than one.

Scheme 2. Reaction Scheme for the Adenosylcobalamin (AdoCbl; R = H) and, Separately, 8-Methoxy-5′-deoxy-adenosylcobalamin (8-MeOAdoCbl; R = MeO) Homolysis in Ethylene Glycol Solution



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 a mixture of $HOCH_2$-CH_2OH-d_0 and $HOCD_2CD_2OH$-d_4 over a 40 °C temperature range of 80−120 °C. Comparing the results to the enzyme tunneling data for AdoCbl-dependent methylmalonyl-CoA mutase (vide supra) reveals that the enzyme-free and enzymic data sets *are identical within experimental error*. The Occam's Razor 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) within experimental error for the H• abstraction both within and outside of the enzyme.[32]

Results and Discussion

The systems studied are shown in Scheme 2: the AdoCbl system, where R = H, and the 8-MeOAdoCbl system, where R = MeO. The AdoCbl thermolysis reaction in ethylene glycol (Scheme 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 by 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 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 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 2). The desired KIE is readily obtained via eq 1

$$\mathrm{KIE} = \frac{k_{\mathrm{aH}}}{k_{\mathrm{aD}}} = \frac{(\text{c-Ado/AdoD})_{d_4\text{solution}}}{(\text{c-Ado/AdoD})_{d_0\text{solution}}} = \frac{r(\mathrm{D})}{r(\mathrm{H})} \qquad (1)$$

where r(H) is the product ratio, c-Ado/Ado-H, observed in nondeuterated ethylene glycol solvent and r(D) is the corre-

(43) There is evidence that indicates that the Co−C bond cleavage and the hydrogen atom abstraction are coupled.[44] Note, however, that our data showing identical tunneling criteria with and without the enzyme indicate substantial adenosyl radical character in the hydrogen atom abstraction reaction.

(44) Padmakumar, R.; Padmakumar, R.; Banerjee, R. *Biochemistry* **1997**, *36*, 3713.

(45) Dybala-Defratyka, A.; Paneth, P. *J. Inorg. Biochem.* **2001**, *86*, 681.

(46) There is some nondefinitive data for QM tunneling in diol dehydratase, ethanolamine, ammonia lyase, and glutamate mutase. See sections S-3 through S-5 of the Supporting Information for further discussion.

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

(49) A small amount of the adenine (~5%) is also observed due to a well-established, competitive, pH-dependent Co−C heterolysis.[51]

(50) The most probable mechanism for the H atom loss reaction after the cyclization step involves H atom abstraction from the cyclized nucleoside radical by the persistent cobalamin radical. The possible disproportionation of the cyclized nucleoside radical is probably never kinetically viable once even a small amount of Co(II)Cbl builds up.[52]

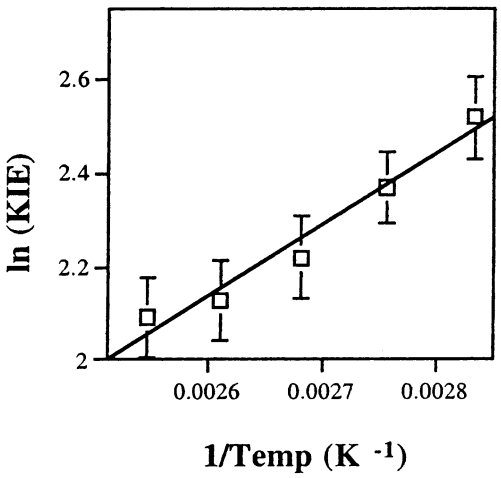

Figure 1. A plot of ln KIE vs 1/*T* for the AdoCbl thermolysis. The activation energy difference and the pre-exponential factor ratio were calculated from the slope and intercept, respectively.

sponding product ratio observed in deuterated ethylene glycol (a derivation is provided in Section S-6, derivations 1 and 2 of the Supporting Information).

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 to 120 °C, temperatures where AdoCbl homolysis proceeds at a convenient rate. The thermolysis reaction proceeded as shown in Scheme 2, exhibiting clean conversion to Co(II)Cbl$^\bullet$ by UV−visible spectroscopy (Figure S1, Supporting Information), plus the expected products by HPLC (Figures S2 and 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[53−55] of ethylene glycol, with a KIE ranging from 8.1 ± 0.7 at 120 °C to 12.4 ± 1.1 at 80 °C, Figure 2. These isotope effects are 45−94% larger than the predicted, maximum primary times secondary GS-ZPE KIEs of 5.5 at 120 °C and 6.4 at 80 °C, respectively, calculated using a version of the Bigeleisen equation: $k_H/k_D \approx e^{[hc(\nu_{CH} - \nu_{CD})]/2k_BT}$ (and 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, $[E_D - E_H] = 3.0 \pm 0.3$ kcal mol^{-1}, was calculated from the slope of a ln KIE vs 1/*T* Arrhenius plot (Figure 1), and the ratio of pre-exponential factors, $A_H/A_D = 0.16 \pm 0.07$, was calculated from the intercept of that plot. Both of these criteria indicate the expected[11b] substantial tunneling in the enzyme-free, Ado$^\bullet$-mediated H$^\bullet$ abstraction reaction.

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 1). The KIEs, activation energy difference, and ratios of pre-experimental factors are all *the same within experimental error* if the true error bars of each measurement are taken into account. The only case where it looks as though there might be a difference beyond experimental error is the (small) A_H/A_D ratio for methylmalonyl-CoA mutase. However, our independent linear-regression analysis (Section 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 published 0.078 ± 0.009),[2] as well as a somewhat different value 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 1 is that *the enzymic and enzyme-free tunneling criteria are the same within experimental error*.

The above findings are important enough that we wanted a second system where we could check our data and the above initial conclusion. In particular, if one can stop Ado$^\bullet$ cyclization (Scheme 2), then one can measure the Ado-H to Ado-D product ratio directly (pathways 2 and 3, Scheme 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$^\bullet$ and 8-MeOAdo$^\bullet$. As desired, 8-MeOAdo$^\bullet$ does not cyclize (does not exhibit path 1, Scheme 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.

Following our previous protocol, 8-MeoAdoCbl was thermolyzed at 10 °C increments from 80 to 120 °C, this time in a mixture of 15% ethylene glycol and 85% ethylene glycol-d_4. Conversion to Co(II)Cbl$^\bullet$ was again observed by UV−visible spectroscopy (Figure 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 S6) and by using eq 2.

$$\mathrm{KIE} = \frac{k_{aH}}{k_{aD}} = \frac{\dfrac{m/z\text{: 282 peak}}{\%\mathrm{glycol}_{d_0}}}{\dfrac{m/z\text{: 283 peak} - 14.46}{\%\mathrm{glycol}_{d_4}}} \qquad (2)$$

The 14.46 term in eq 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.

Even in 85% ethylene glycol-d_4 the peak in the mass spectrum corresponding to 8-MeOAdo-H is larger than the peak corre-

(51) Hay, B. P.; Finke, R. G. *J. Am. Chem. Soc.* **1986**, *108*, 4820.
(52) Daikh, B. E.; Finke, R. G. *J. Am. Chem. Soc.* **1992**, *114*, 2938.
(53) The hydrogen abstraction does occur from the C−H (and not the O−H) bond as expected by the literature[54,55] on methyl radical abstraction from MeOH. The expected C−H reaction was also demonstrated in the present work by a control experiment using ethylene glycol-d_6 (section S-1 in the Supporting Information).
(54) Phibbs, M. K.; Darwent, B. d. *Can. J. Res.* **1950**, *28B*, 395.
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(57) Frei, H.; Ha, T.-K.; Meyer, R.; Guenthard, H. H. *Chem. Phys.* **1977**, *25*, 271.
(58) The required extrapolation of our ln KIE vs 1/*T* plot to lower temperatures rather than measured experimentally leads to an estimated lower limit of the KIE of 29 at 20 °C, similar to the value of 35.6 reported for methylmalonyl-CoA mutase.[2] Alternatively, an extrapolation of the enzymic data to higher temperatures leads to an estimated enzymic KIE of 12.7 at 80 °C, again within experimental error of our enzyme-free KIE of 12.4 ± 1.1. We have also examined a system, *β*-neopentylcobalamin, where thermolysis occurs in the same temperature range in which the enzymic tunneling data was obtained.[59] That system, again, shows no difference within experimental error for the three tunneling criteria vs those for methylmalonyl-CoA mutase.[59]
(59) Doll, K. M.; Finke, R. G. *Inorg. Chem.* **2003**, *42*, 4849−4856.
(60) Doll, K. M.; Fleming, P. E.; Finke, R. G. *J. Inorg. Biochem.* **2002**, *91*, 388.
(61) Hartshorn, A. J.; Johnson, A. W.; Kennedy, S. M.; Lappert, M. F.; MacQuitty, J. J. *J. Chem. Soc., Chem. Commun.* **1978**, 643.

Table 1. Observed KIE, Activation Energy Difference, and Pre-exponential Factor Ratio for Our Nonenzyme 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 ± 0.028)[d]
ethanolamine ammonia lyase[67,68]		(3.1 ± 1.1)[e]	(0.038 ± 2.13)[e]
glutamate mutase[69,70]	28−35 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	6.8 at 10 °C 6.4 at 20 °C	1.2	1.0
GS-ZPE 1° × 2°	9.5 at 10 °C[g] 8.9 at 20 °C[g] 6.4 at 80 °C[g] 5.5 at 120 °C[g]	1.2	1.0

[a] Values are extrapolated from the higher temperature data. [b] The larger error bars for this 8-MeOAdoCbl data set are due to what proved to be 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 analyses of the literature data set.[2] See the Supporting Information, Section S-2, for further details. [e] These data were calculated from data in the literature.[68] See the Supporting Information, Section S-4 for further details. [f] NA = not available in the literature. [g] The GS-ZPE 1° and 2° KIE = [(1° KIE) × 1.15 × 1.1^2].

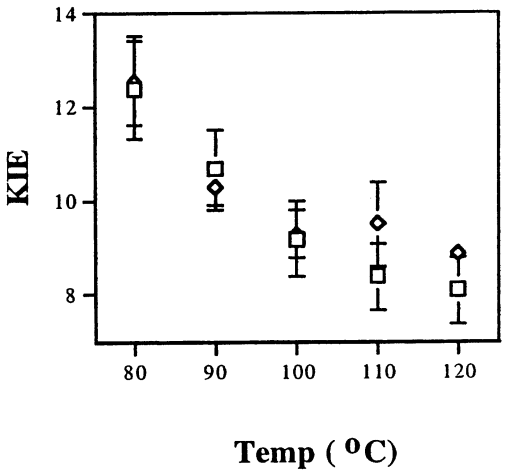

Figure 2. A plot of the observed kinetic isotope effects (KIEs) of the hydrogen abstraction from ethylene glycol vs temperature: Ado• (□), 8-MeOAdo• (◇). The results show that both sets of data are identical within experimental error.

sponding to 8-MeOAdo-D (Figure 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 2). 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 S-7). 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; that is, the 8-MeoAdoCbl/HPLC−MS system was not able to deliver a more precise data set as we had hoped. Nevertheless, the data obtained for 8-MeOAdoCbl are within experimental error of the values obtained for AdoCbl (Figure 2 and Table 1). The results are again indicative of the expected[11b] substantial QM tunneling in the enzyme-free, 8-MeOAdo• H atom abstraction reaction. Significantly, they show no difference within experimental error vs the tunneling data for the enzyme methylmalonyl-CoA mutase (Table 1).

The above data complete the first experimental test of the hypothesis of enzyme-enhanced tunneling. Perhaps most importantly, they demonstrate a definitive method to test the enhanced hydrogen tunneling hypothesis, the approach summarized in Scheme 1. Our results reveal that, at least in the H• abstraction reaction by Ado•, the Occam's Razor interpretation of the data is that 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.

Three 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 the 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 suggests that this is not an issue as does our finding of identical results within experimental

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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 Ado• is special in its degree of tunneling vs a simple alkyl R•. Thus far, the answer is that Ado• is not special.[57,58] A third caveat is that one would like an even more precise enzyme-free data set so that the finding of *no enzyme-enhancement of tunneling within experimental error* could be tightened beyond the present $\Delta(E_D - E_H) = 0.54 \pm 0.36$ kcal/mol and $\Delta(A_H/A_D) = 0.08 \pm 0.08$ (1 σ error bars) obtained using the enzymic, methylmalonyl-CoA mutase minus the enzyme-free AdoCbl data in rows 3 and 1, respectively, of Table 1.

Finally, although this first experimental test of Klinman's hypothesis strongly suggests that at least H• abstracting, B_{12}-dependent enzymes have not evolved to enhance QM tunneling, more studies are needed of different enzymes, using the method of Scheme 1 plus the Kreevoy criteria, to know whether the "no-enhancement" results herein are more generally applicable. It is quite 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} s^{-1} [47,80] since these rates are already fast enough for the fairly slow[81−84] turnover rates for B_{12}-dependent enzymes, ca. 22−600 s^{-1}.[75,85] Nevertheless, the starting hypothesis for future work now has to be the main finding from this work: there is no *experimentally* documented case where enzyme-enhanced QM tunneling has been unequivocally demonstrated, and there is one case (the present work) where the data show that there is no enhancement of QM tunneling within experimental error. Instead, the Occam's Razor interpretation of the data is that 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 require an unchanged barrier width, height, and rate for both the enzyme and enzyme-free H abstraction 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]

In a now-completed second study,[59] we have expanded analogous KIE vs temperature studies of R• abstraction from ethylene glycol-d_0 and -d_4 over a 110 °C temperature range. That study provides relatively precise, enzyme-free data of $(E_D - E_H) = 3.15 \pm 0.08$ kcal mol^{-1} and $A_H/A_D = 0.13 \pm 0.02$ and reduces the $\Delta(E_D - E_H)$ to 0.39 ± 0.20 kcal/mol and the $\Delta(A_H/A_D)$ to -0.048 ± 0.038 (again 1σ error bars).[59] Those results fortify the preliminary findings and conclusions of the present work. They also fortify the value of this first study demonstrating the significance, success, and pitfalls (e.g., the need for highly precise enzymic as well as nonenzymic data) of the approach summarized in Scheme 1.

We discuss in more detail in our follow-up study[59] the question of the possible broader generality of the "no enzyme-enhancement of hydrogen tunneling" finding herein, including a discussion of Siebrand's important paper examining the current experimental and theoretical evidence for what they call the "protein-squeezing" (i.e., protein-reduced tunneling width) hypothesis.[86] Notably, Siebrand concludes that "...the hypothetical protein-squeezing mechanism...is rejected on theoretical and experimental grounds."[86]

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)), and 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 nanopure 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 × 4.6 mm Alltech C-18 reversed-phase column. HPLC−MS was done with an HP 1100 HPLC with a 200 mm × 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 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 running on a Power Macintosh 5400/120.

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(86) (a) Siebrand, W.; Smedarchina, Z. *J. Phys. Chem.*, submitted for publication, 2003; we thank Dr. Siebrand for sharing a preprint. This paper looks critically at the experimental and theoretical "evidence" for the protein-enhanced tunneling hypothesis (i.e., the "protein-squeezing" hypothesis).[86b] Siebrand and Smedarchina find problems in both the experimental[86b] and theoretical work supposedly supporting the protein-squeezing mechanism. They conclude that "...the hypothetical protein squeezing mechanism leads to very short (physically unreasonable) distances combined with low anharmonicities, and is rejected on theoretical and experimental grounds."[86a] They also argue that generally flexible proteins are "...ill-equipped to cause strong local compression."[86a] They provide an alternative mechanism consisting of charge transfer along with a coupled H^+-transfer and detail how that enhances tunneling by shortening the H^+-transfer distance and increasing the tunneling mode anharmonicity, the two most important parameters in their tunneling model.[86a] (b) Knapp, M. J.; Rickert, K. W.; Klinman, J. P. *J. Am. Chem. Soc.* **2002**, *124*, 3865. Siebrand and Snedarchina[86a] show that there is no statistically valid difference in the data in this paper for mutant vs wild-type enzyme (because of the too-narrow temperature range over which the data could be obtained), thereby negating the claimed support in this paper for the putative protein-squeezing mechanism.

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 °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.

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 three times by freeze/evacuate/refill with argon/thaw cycles) was added with a syringe, giving a $\sim 2 \times 10^{-4}$ 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 $\sim 7 \times 10^{-5}$ M. The cuvettes were brought out of the drybox and into the darkroom for thermolysis. Four to thirteen thermolyses (see Table S1) in both ethylene glycol-d_0 and ethylene glycol-d_4 were carried out at temperatures of 80 (8 days), 90 (94 h), 100, 110, 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 S1), and clean isosbestic points at 336, 394, 486, and 586 nm were observed. The spectra indicate direct and complete conversion to Co(II)Cbl$^{\bullet}$.[47] The $\sim 2.4 \times 10^{-4}$ 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 S2 and 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 ~8.9 min, ~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 ~8.5 min, ~79% total peak area), and a peak corresponding to 5′-deoxyadenosine (Ado-D; retention time ~12.5 min, ~2.3% total peak area).[87] 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 S-6 in the Supporting Information).

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 three times by freeze/evacuate/refill with argon/thaw cycles) was added to the vial. The Schlenk cuvettes were filled with either 0.75 or 0.85 mL ethylene glycol-d_4 (also degassed), then 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 $\sim 4 \times 10^{-4}$ M solution in which the solvent was either 75% or 85% ethylene glycol-d_4 and the remaining solvent was ethylene glycol-d_0 (a lower concentration of $\sim 1.2 \times 10^{-4}$ M was tried, but the HPLC−MS signal was substantially better using the 3-fold higher concentration). 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 (154 h), 90 (105 h, 40 min), 100 (33 h), 110 (21 h), and 120 °C (4 h, 48 min). UV−visible spectra were taken at regular intervals (Figure S4). Isosbestic points at 338, 392, 486, and 585 nm were observed, indicating complete conversion to Co(II)Cbl$^{\bullet}$. 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.[88] Peaks were identified by the molecular ion peaks in their mass spectrum. A typical HPLC trace (Figure 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 S6) and from using eq 2. Because the *m*/*z* ratio of 282 corresponds to $[8\text{-MeOAdo-H} + H^+]^+$ and the *m*/*z* ratio of 283 corresponds to $[8\text{-MeOAdo-D} + H^+]^+$, the KIE can be calculated from the *m*/*z*: 282 peak and the *m*/*z*: 283 peak. The *m*/*z*: 283 peak must be corrected for contribution from heavier isotopes in the nondeuterated product; therefore, 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 (eq 2).

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Supporting Information Available: 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 S-1: A Control Experiment 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 S-2: Discussion and Plot of ln KIE vs 1/*T* using the data from Banerjee. Section S-3: Discussion of Tunneling Evidence in Diol Dehydratase. Section S-4: Discussion of Ethanolamine Ammonia Lyase including a Plot of ln *k* vs 1/*T* for Both Protiated and Deuterated Substrates. Section S-5: A

(87) Two of the HPLC traces contained a small unassigned peak (retention time ~7.7 min). It did not have a UV−visible signal at 525 nm, indicating that it is not a cobalamin. It is suspected, but not proven, that it is a nucleoside formed by the cycloadenosyl radical that has not completely converted to c-Ado. Because this peak was only present in two out of 34 samples, and since there was no corresponding peak in the deuterated ethylene glycol system, chromatograms with this peak were not used in the calculation of the ratio of c-Ado/Ado-H.

(88) 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.

Short Discussion of Tunneling Evidence in Glutamate Mutase. Section S-6: Derivation of Equation 1. Section S-7: Relation of $E_D - E_H$ and A_H/A_D to a ln KIE vs $1/T$ plot. Figure S1: The UV−visible spectra of an AdoCbl thermolysis reaction at 90 °C. Figure S2: An HPLC chromatogram for an ethylene glycol-d_0 reaction at 80 °C. Figure S3: An HPLC chromatogram for an ethylene glycol-d_4 reaction at 80 °C. Figure S4: The UV−visible spectra of an 8-MeOAdoCbl thermolysis reaction at 90 °C. Figure S5: The representative HPLC−MS from a 100 °C thermolysis reaction of 8-MeOAdoCbl in 85% ethylene glycol-d_4. Figure S6: A representative comparison of the corresponding ratios of 8-MeOAdo-H (281.9) and 8-MeOAdo-D (282.9). Figure S7: A plot of the KIE vs $1/T$ for the 8-MeO-AdoCbl thermolysis. Table S1: The ratio c-Ado/Ado-H in post-thermolysis Ado-B_{12} in ethylene glycol-d_0. Table 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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