How B_6 Helps B_{12}: The Roles of B_6, B_{12}, and the Enzymes in Aminomutase-Catalyzed Reactions

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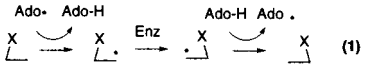
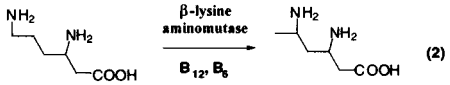
Received May 17, 2000

Coenzyme B_{12} (5′-deoxyadenosylcobalamin) is an essential cofactor in a wide range of biochemical transformations.[1] The majority of B_{12}-dependent reactions involve the vicinal interchange of a group X and a hydrogen atom, via radical intermediates:



The coenzyme is thought primarily to act as a source of 5′-deoxyadenosyl radicals (Ado•), which serve to activate the substrate (eq 1). This mechanism implies that a crucial step in the transformation is the 1,2-shift of a migrating group X to an adjacent radical center, a reaction known generally to be associated with a high activation energy. Previous work has shown that this intrinsically difficult step can be facilitated when (a) the migrating group is unsaturated,[2a−c] (b) the migrating group is (partially) protonated,[2b−d] or (c) an adjacent substituent is (partially) deprotonated.[2e,f]

In the present investigation, we turn our attention to a class of B_{12}-dependent enzymes which catalyze reactions involving a migrating amino (NH_2) substituent, namely the aminomutases. These enzymes usually act on amino acids (e.g., β-lysine) to prepare the relevant carbon skeleton for subsequent cleavage into easily metabolized products:[3]



The B_{12}-dependent aminomutases also require pyridoxal 5′-phosphate (PLP), a biologically active form of vitamin B_6, for their normal function. The main focus of the current investigation is to probe the reason for, and the mechanism of, this requirement.

The B_{12}-dependent reactions typically proceed with a k_{cat} between 40 and 150 s^{-1}.[4] Experimental rate data for lysine 2,3-aminomutase are consistent with this range.[5] We estimate[6] that the barrier for the rate-limiting step in B_{12}-catalyzed 1,2-amino shifts should lie between approximately 50 and 75 kJ mol^{-1}. The barrier for the radical rearrangement step must therefore fall within or below this range. Due to difficulties inherent in probing radical mechanisms experimentally, it is advantageous to examine the barriers associated with plausible pathways using quantum chemical techniques.[7]

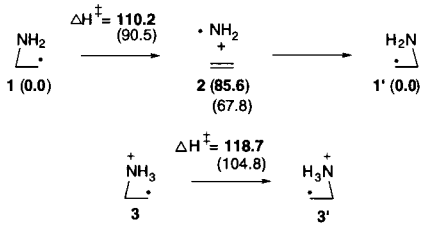
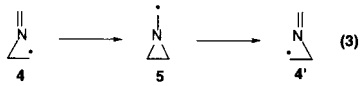


Figure 1. RMP2 (boldface) and G3(MP2)-RAD(p) relative energies (kJ mol^{-1}) for species involved in two pathways for the degenerate rearrangement of the 2-aminoethyl radical.

To determine the propensity of an amine group to migrate to an adjacent radical center, we have initially examined the degenerate rearrangement of the 2-aminoethyl radical (Figure 1). Although a transition structure describing a concerted migration between **1** and **1′** could not be located, a stepwise pathway (fragmentation-recombination) involving separation into the amino radical and ethylene (**2**), followed by recombination of the fragments, was characterized. This pathway is associated with a barrier of 110.2 kJ mol^{-1}. As with the fragmentation-recombination mechanisms of other B_{12}-assisted rearrangements,[2] this mechanistic alternative appears to be associated with a barrier too high to be considered biologically relevant. We have previously found that protonation of the migrating group facilitates many 1,2-shifts.[2] However, the calculated barrier (118.7 kJ mol^{-1}) for the intramolecular rearrangement for a migrating protonated amino substituent (**3** → **3′**, Figure 1) is even higher than that for the fragmentation-recombination alternative. With the avenues of both direct migration and migration assisted by protonation closed, nature is forced to find another way to render the 1,2-shift of an amino group feasible. This is apparently accomplished through judicious use of vitamin B_6.

PLP is thought to form an imine link with a reactive amino group of the substrate.[9] Experimental evidence supporting this interaction has been obtained through electron spin resonance studies.[5,10] In the context of the B_{12}-dependent aminomutases, the mechanism of action proposed for PLP entails the introduction of unsaturation to the migrating group, a characteristic known to facilitate 1,2-shifts.[2]

We have examined the effect of formation of an imine with PLP by considering the degenerate rearrangement of the 2-(*N*-methylidine)ethyl radical (**4**):[11]



The introduction of imine functionality causes the reaction to proceed via a three-membered cyclic intermediate (**5**), as discussed

(1) See, for example: Golding, B. T.; Buckel, W. In *Comprehensive Biological Catalysis*; Sinnott, M. L., Ed.; Academic Press: London, 1997; Vol. III, pp 239−259, and references therein.

(2) (a) Smith, D. M.; Golding, B. T.; Radom, L. *J. Am. Chem. Soc.* **1999**, *121*, 1037−1044. (b) Smith, D. M.; Golding, B. T.; Radom, L. *J. Am. Chem. Soc.* **1999**, *121*, 1383−1384. (c) Smith, D. M.; Golding, B. T.; Radom, L. *J. Am. Chem. Soc.* **1999**, *121*, 9388−9399. (d) Golding, B. T.; Radom, L. *J. Am. Chem. Soc.* **1976**, *98*, 6331−6338. (e) Smith, D. M.; Golding, B. T.; Radom, L. *J. Am. Chem. Soc.* **1999**, *121*, 5700−5704. (f) Smith, D. M.; Golding, B. T.; Radom, L. Submitted for publication.

(3) Baker, J. J.; Stadtman, T. C. In *B_{12}*; Dolphin, D., Ed.; Wiley: New York, 1982; Vol. II, pp 203−232.

(4) (a) Bachovchin, W. W.; Eager, R. G., Jr.; Moore, K. W.; Richards, J. H. *Biochemistry* **1977**, *16*, 1082−1092. (b) Meier, T. W.; Thoma, N. H.; Leadlay, P. F. *Biochemistry* **1996**, *35*, 11791−11796. (c) Babior, B. M. In *B_{12}*; Dolphin, D., Ed.; Wiley: New York, 1982; Vol. 2, pp 263−287. (d) Holloway, D. E.; Marsh, E. N. G. *J. Biol. Chem.* **1994**, *269*, 20425−20430.

(5) (a) Frey, P. A.; Reed, G. H.; Moss, M. L.; Petrovich, R. M.; Ballinger, M. D.; Lieder, K. W.; Wu, W.; Chang, C. H.; Bandarian, V.; Ruzicka, F. J.; LoBrutto, R.; Beinert, H. In *Vitamin B_{12} and B_{12}—Proteins*; Kräutler, B., Arigoni, D., Golding, B. T., Eds.; VCH: 1998; pp 435−446, and references therein. (b) Lysine 2,3-aminomutase utilizes an iron−sulfur cluster and *S*-adenosylmethionine to abstract hydrogen in the initial step rather than coenzyme B_{12}. However, the radical rearrangement mechanism is likely to be the same for all aminomutases regardless of the hydrogen-abstraction pathway.

(6) Calculated by techniques suggested previously. See: George, P.; Glusker, J. P.; Bock, C. W. *J. Am. Chem. Soc.* **1997**, *119*, 7065−7074.

(7) Unless otherwise noted, theoretical energies reported in this study have been obtained with RMP2 using the G3MP2Large basis set on structures optimized at the B3-LYP/6-31(d) level. The accuracy of this technique is benchmarked through comparisons with the results obtained with G3(MP2)-RAD(p) (a technique based on high-level G3(MP2) and modified to yield improved results for radicals) for smaller models.[8]

(8) Details of our calculations and an expanded discussion will appear in a full paper.

(9) See, for example: Lehninger, A. L.; Nelson, D. L.; Cox, M. M. *Principles of Biochemistry*; Worth Publishers: New York, 1993.

10.1021/ja001651y CCC: $19.00
Published on Web 09/29/2000

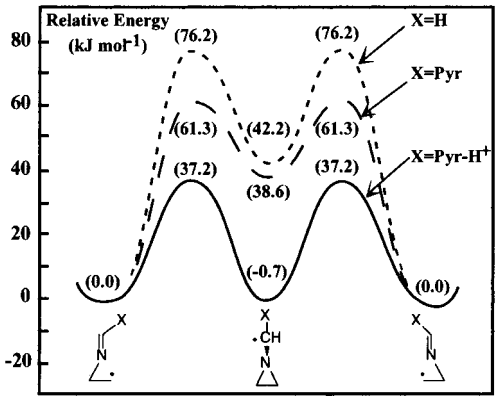
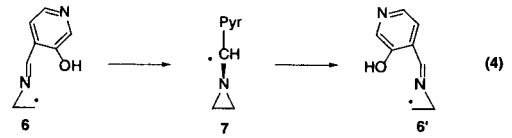


Figure 2. Schematic energy profile for the degenerate rearrangement of substituted 2-(*N*-methylidine)ethyl radicals where X = H (**4**), Pyr (pyridin-3-ol, **6**) or Pyr-H$^+$ (pyridin-3-ol protonated at the ring nitrogen).

for other unsaturated migrating groups.[2] This intermediate lies 42.2 kJ mol^{-1} higher in energy than the open-chain reactant (**4**) and the barrier to its formation is 76.2 kJ mol^{-1}.[12] While the energy requirement for this reaction is significantly lower than those described in Figure 1, it is still slightly larger than our estimated suitable range. We have previously found that (partial) protonation at a double bond reduces the barrier height in models of several related reactions.[2] However, protonation does not appear to be the answer for the **4** → **4′** rearrangement.[8,13]

To investigate whether PLP has effects beyond the introduction of imine functionality to the migrating group, the model system was extended to include a simplified ring (**6**):



The substituted cyclic intermediate formed by this modification (**7**) is stabilized to a greater extent than its unsubstituted counterpart (**5**), relative to the appropriate reactant (Figure 2), and the barrier to ring closure (**6** → **7**) is reduced to 61.3 kJ mol^{-1}. However, because the energy effect is small, it is tempting to suggest that the dominant role of PLP is the introduction of a double bond to the migrating group.

On the other hand, since pathways with barriers much less than 60 kJ mol^{-1} have been identified for many other B_{12}-dependent rearrangements,[2] it is desirable to try to unveil a mechanism by which the enzyme might further reduce the rearrangement barrier. Led by these previous studies, we investigated protonation of the substituent adjacent to the migrating group, specifically at the nitrogen of the pyridin-3-ol ring (eq 4). Protonation at this site extensively stabilizes the cyclic intermediate (**7**-**H**$^+$), which is found to lie 0.7 kJ mol^{-1} below the relevant reactant radical. As a result of this stabilization, the barrier for ring-closure (**6**-**H**$^+$ → **7**-**H**$^+$) is strikingly reduced to just 37.2 kJ mol^{-1} (Figure 2).[14,15]

Taken together, the above results suggest that the first important contribution of B_6 to facilitating high-energy 1,2-amino shifts is the introduction of a double bond into the migrating group. However, the rate of the ring-closing/ring-opening mechanism that ensues is retarded by the relatively high energy of the three-membered cyclic intermediate. Although effects such as electron donation by the adjacent nitrogen lone pair or slight electron withdrawal by the pyridine ring are expected to stabilize this cyclic intermediate, these effects alone do not yield a reaction barrier low enough to fully explain the observed catalysis. However, when the electron-withdrawing capacity of the ring is increased through protonation, the stability of the cyclic intermediate is greatly enhanced and the reaction barrier is significantly reduced.

We propose that it is actually the cooperation of electron donation (by the nitrogen lone pair of the three-membered ring) and electron withdrawal (in particular by the protonated pyridoxal ring) acting upon the radical center that causes the notable stabilization predicted for the cyclic intermediate. This synergistic combination of electron-donor and -acceptor substituents has been observed in many other radicals and is commonly referred to as captodative stabilization.[16] Application of our previously proposed partial protonation concept[2] suggests that weak hydrogen bonding to the pyridine ring (as an alternative to full protonation) may also provide sufficient captodative stabilization of the cyclic intermediate. The captodative stabilizing role of PLP supports hypotheses that the pyridoxyl functionality acts as an "electron sink".[9] On the other hand, recent theoretical studies found no evidence that protonation at the pyridine ring increases the rate of decarboxylation,[17] suggesting that the responsibilities of the pyridoxyl ring may vary with the nature of the reaction.[18]

Although the 1,2-shift of an amino group appears to be a demanding task, our calculations show that the rearrangement may be efficiently accomplished as a result of an intricate relationship between the enzyme and its cofactors. Thus, we believe that coenzyme B_{12} is responsible for activating the substrate by removal of a hydrogen atom. Vitamin B_6 (PLP) introduces a seemingly essential double bond into the migrating group, as well as imparting the potential for the reaction intermediate to be captodatively stabilized. The enzyme itself holds all the components in place and provides an environment in which the pyridine nitrogen can be fully (or partially) protonated. This cooperative action of the enzyme and cofactors is able to mediate an otherwise difficult reaction.

Acknowledgment. We gratefully acknowledge the ANU Supercomputing Facility for generous grants of computer time. S.D.W. thanks NSERC for financial support.

JA001651Y

(10) Ballinger, M. D.; Frey, P. A.; Reed, G. H.; LoBrutto, R. *Biochemistry* **1995**, *34*, 10086−10093.

(11) These radicals have been studied experimentally. See: Danen, W. C.; West, C. T *J. Am. Chem. Soc.* **1974**, *96*, 2447−2453.

(12) For comparison, the G3(MP2)-RAD(p) relative energies for **5** and the barrier height are 51.8 and 77.8 kJ mol^{-1}, respectively.

(13) Protonation at nitrogen does not reduce the barrier for reaction 3, while protonation at carbon leads to the 2-azabut-2-ene radical cation.

(14) We note that protonation at the pyridine nitrogen is preferred over protonation at the imine nitrogen by 33 kJ mol^{-1}, presumably because it avoids disrupting the intramolecular hydrogen bond to the hydroxyl group.

(15) If the active site is largely sequestered from water, as found for other B_{12}-dependent enzymes, then the protonation site will primarily be determined by the local structure of the enzyme. We find that it is protonation at the pyridine nitrogen that is most effective in catalyzing the reaction.

(16) For a review, see: Viehe, H.-G.; Janousek, Z.; Merényi, R.; Stella, L. *Acc. Chem. Res.* **1985**, *18*, 148−154.

(17) Bach, R. D.; Canepa, C.; Glukhovtsev, M. N. *J. Am. Chem. Soc.* **1999**, *121*, 6542−6555.

(18) PLP-dependent decarboxylases involve carbanionic intermediates, while PLP-dependent aminomutases catalyze radical reactions.