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Probing the Mechanism of Coenzyme B_{12}: Synthesis, Crystal Structures, and Molecular Modeling of Coenzyme B_{12} Model Compounds

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Coenzyme B_{12} is an organometallic compound that catalyzes biological rearrangement reactions. Homolytic cleavage of the unusual cobalt-carbon bond in the coenzyme initiates the free-radical reaction. In an attempt to understand the factors that might be important in the mechanism, we synthesized a series of model complexes [LCo{(DO)(DOH)bn}R]$^+$ with a folded equatorial ligand and determined the crystal structures. Semi-empirical calculations with these and other model compounds provide evidence for a transelectronic influence; when the Co–N bond is shortened, the Co–C bond lengthens. These results are compared to density functional calculations carried out by others.

Keywords: B_{12}, coenzyme B_{12}, deoxyadenosylcobalamin

INTRODUCTION

The cobalamins are fascinating molecules. They are organometallic compounds that function as biological catalysts. Although liver extract taken orally had been discovered as a cure for pernicious anemia in 1926,[1] it was

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not until 1948 that a red crystalline compound, vitamin B_{12}, was isolated from liver by workers at Merck and at Glaxo.[2] When Hodgkin and co-workers[3] determined the structure of vitamin B_{12} (cyanocobalamin), they found the Co(III) ion coordinated to five nitrogen donor atoms, four N donors from a macrocyclic corrin ligand and one from a benzimidazole pendant from the corrin (Figure 1). The equatorial part of the corrin system is similar to the

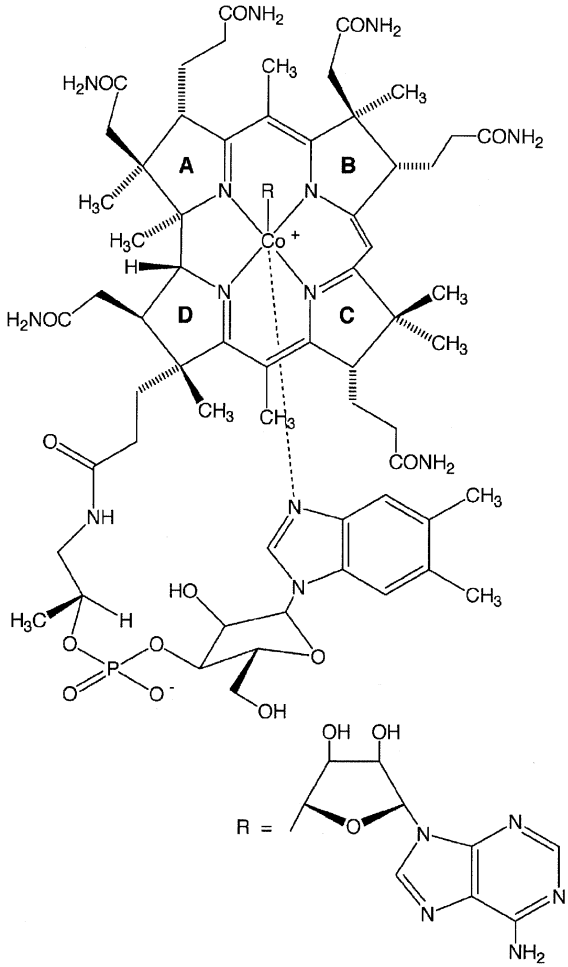


FIGURE 1 Structural formula of the cobalamins. In vitamin B_{12}, R = CN; in adenosylcobalamin (AdoCbl), R = 5′-deoxyadenosyl; in methylcobalamin (MeCbl), $R = CH_3$.

porphyrin ring, except that the five-membered pyrrole-like rings are partially reduced and a bridging methine group is "missing." The direct connection between the A and D rings on the "western" side interrupts the π system, so that the corrin macrocycle is not aromatic. In addition, since there is a bond between two tetrahedral carbon atoms as part of the equatorial ring, the corrin ring cannot be planar. A long side chain on one of the pyrrole-like rings is linked to the nucleotide base 5,6-dimethylbenzimidazole (DMB), which contributes the fifth nitrogen donor atom. The sixth ligand in vitamin B_{12} is the cyano group (an artifact of the isolation chemistry). In the two naturally occurring cobalamins, the sixth ligand is either 5′-deoxyadenosyl or methyl.

The structure of adenosylcobalamin (AdoCbl), or coenzyme B_{12}, also determined by Hodgkin,[4] revealed the naturally occurring cobalt-carbon bond to the 5′-deoxyadenosyl group shown in Figure 1. It is this Co–C bond that is now known to be responsible for the catalytic activity of the B_{12} cofactors. The structure of methylcobalamin[5] (MeCbl) is similar to AdoCbl, with a methyl group bonded to the cobalt instead of the 5′-deoxyadenosyl group. The cobalamins contain one of only two types of metal-carbon bonds found in nature, the other being a methylnickel intermediate in carbon monoxide dehydrogenase.[6]

Recently, the structures of four B_{12}-dependent enzymes have been obtained: methionine synthase,[7] methylmalonyl-CoA mutase,[8] glutamate mutase,[9,10] and diol dehydratase.[11,12] In MeCbl and Class I AdoCbl enzymes, the typical pattern appears to be the substitution of an imidazole ligand (the side chain of a histidine residue from the protein) for the DMB ligand. In Class II AdoCbl enzymes, the DMB is retained as the axial nitrogen-donor ligand (N_{ax}).[13]

For both B_{12} cofactors, Co-C bond cleavage is central to the mechanism. In methyltransferases such as methionine synthase, MeCbl undergoes heterolytic cleavage, forming a Cob(I)alamin and a methyl cation. The methyl group is donated to homocysteine, yielding methionine. In AdoCbl the cleavage is homolytic, forming Cob(II)alamin and 5′-deoxyadenosyl radical.[14] In the human enzyme methylmalonyl-CoA mutase, the 5′-deoxyadenosyl radical abstracts a H atom from the substrate methylmalonyl-CoA. The radical substrate then undergoes a 1,2-rearrangement reaction and the H atom is added back to produce succinyl-CoA. Finally, the 5′-deoxyadenosyl radical recombines with Co(II)alamin to regenerate the catalyst.[15] The enzyme facilitates a spectacular 10^{12}-fold increase in the rate of Co–C bond homolysis.[15] In an effort to learn more about the factors that may destabilize the cobalt-carbon bond, many workers have synthesized organocobalt compounds as models for the cobalamins, especially cobaloximes and Costa compounds.[16]

The equatorial corrin ring in the cobalamins is quite flexible; it folds upward, like the wings of a butterfly, toward the carbon ligand (R). The fold line bisects the corrin ring from west to east, running from the center of the direct connection between rings A and D through the Co to the methine group

on the eastern side. The fold angles depend upon the bulk of the two axial ligands R and L, where L is the axial N-donor ligand.[5,17,18] In a series of cobalamins, the shorter the Co–N_{ax} distance, the greater the upward folding of the corrin ring.[19] For both cobaloxime and Costa model compounds, the larger the ligand L the greater the degree of folding and the weaker the Co–C bond.[16] This steric *trans* influence was suggested by Halpern[20] as a contribution to Co–C bond cleavage in AdoCbl, and the reason why the corrin ring (rather than the porphyrin ring) is the equatorial ligand in coenzyme B_{12}.

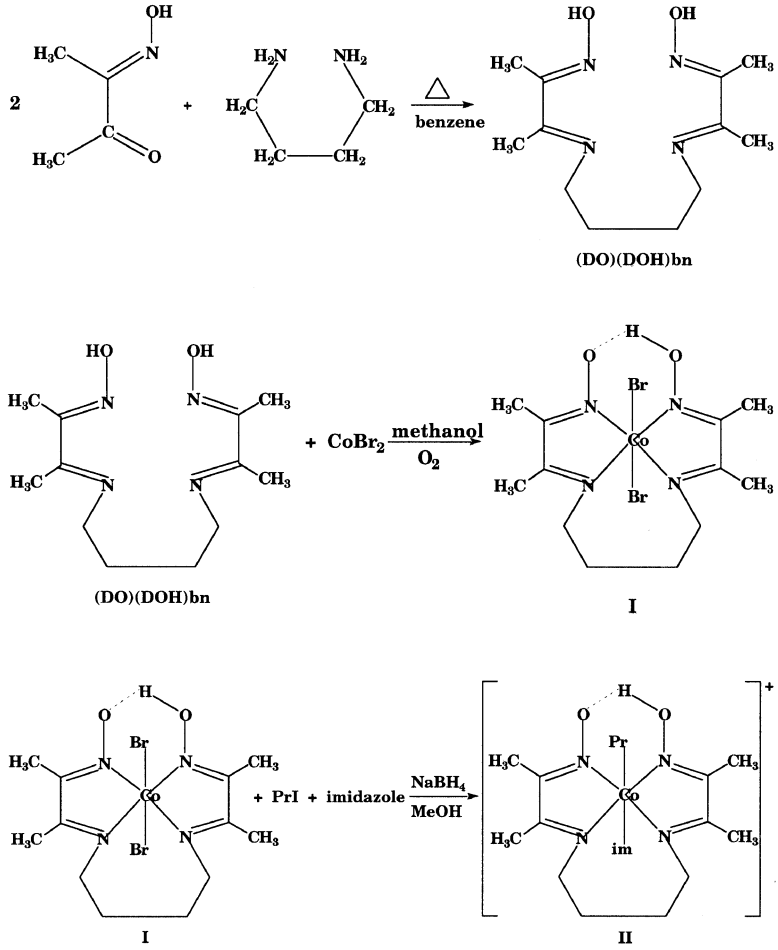
Another aspect of the structure of AdoCbl that may pertain to the mechanism of Co–C bond cleavage is the widening of the Co–C_α–C_β angle in the 5′-deoxyadenosyl ligand from the expected tetrahedral angle to 121–124°.[21,22] This strained angle appears to result from steric interaction between the carbon ligand and the equatorial corrin ring.[23] Pratt[24,25] has suggested that further distortion of the 5′-deoxyadenosyl ligand by the protein (either lengthening the Co–C bond or additional widening of the Co–C_α–C_β angle) could be effective in breaking the Co–C bond.

SYNTHESIS OF ORGANOCOBALT MODEL COMPOUNDS

We have synthesized a series of organocobalt complexes in which the equatorial ligand has a built-in fold, or "buckling" in order to mimic the nonplanar, folded corrin ring.[26,27] The synthetic method (Scheme 1) for these cobaloxime derivatives involves a Schiff base condensation reaction to form a macrocyclic ring, which coordinates to Co(II) through four imino N donor atoms. The complex is easily oxidized in air to produce the Co(III) complex Co{(DO)(DOH)bn}Br_2 (**I**), where (DO)(DOH)bn is the equatorial ligand N^2,$N^{2'}$-butanediylbis(2,3-butanedione 2-imine 3-oxime). Sodium borohydride reduces Co(III) to the Co(I) "supernucleophile," which then displaces I^- from an alkyl iodide to yield the Co–C bond. The latter reaction is conducted in the presence of a base such as imidazole, which then coordinates to the Co(III) ion. These "Wooster analogues," abbreviated $[LCo\{(DO)(DOH)bn\}R]^+$ (**II**), contain a 15-membered macrocyclic ring closed by a hydrogen bond between the two oxime groups. In formal terms, the **II** complexes contain a Co(III) ion coordinated to an R group with charge -1 and a macrocyclic ring with charge -1, giving an overall charge on the complex of $+1$.

STRUCTURE OF WOOSTER ANALOGUES

There are some interesting deviations from octahedral geometry in **II** because of the characteristics of the equatorial ligand. The butane bridge forms an unusual seven-membered chelate ring, the hydrogen bond closes a six-membered chelate ring, while the other two chelate rings have five atoms. In **II** with L = imidazole and R = ethyl (Figure 2), the N(2)–Co–N(3) angle is



SCHEME 1 Optimized synthesis of B_{12} analogues [LCo{(DO)(DOH)bn}R]PF_6. R is an organic ligand with a carbon atom bonded to Co(III). L is H_2O or imidazole (im).

widened to 104° in the seven-membered ring, the N(1)–Co–N(4) angle in the six-membered ring is 94°, and in the two smallest rings the angles are compressed to 80° and 82°. The Co-N(2) and Co–N(3) bonds are lengthened to accommodate the seven-membered chelate ring. Likewise, in the corrin ring of AdoCbl, the N–Co–N angle for the single five-membered chelate ring is smaller than the N–Co–N angles of the other three six-membered chelate rings.[21,22]

As anticipated, the unusual seven-membered chelate ring in the Wooster complexes adds flexibility and steric bulk to the equatorial ligand. The

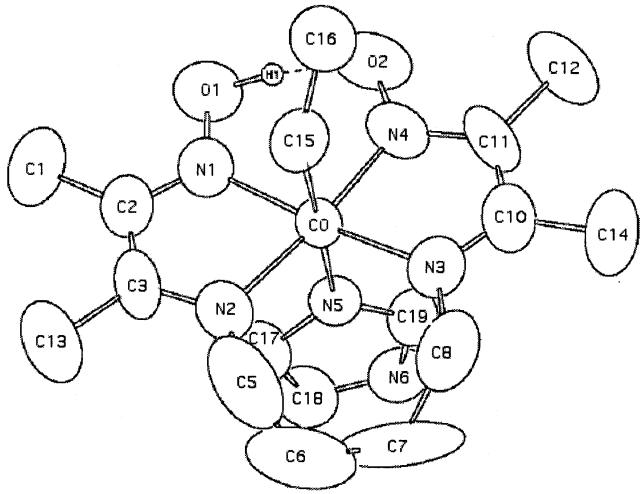


FIGURE 2 The structure of [(imidazole)Co{(DO)(DOH)bn}(ethyl)]$^+$ (molecule 1) showing atom numbering and thermal ellipsoids at the 50% probability level. The oxime H atom that closes the 15-membered macrocyclic ligand with a hydrogen bond is shown. Other H atoms are omitted for clarity.

relatively high thermal motion of the carbon atoms of the butyl bridge [C(5)-C(6)-C(7)-C(8)] is indicated by the size of the thermal ellipsoids in Figure 2. As shown in Table 1, the flexibility of the butane bridge allows the equatorial ligand to fold in either direction. The fold line bisects the seven-membered chelate ring, runs through the Co atom, and bisects the O–H $\cdots$ O hydrogen bond. Apparently, the relative size of R and L controls the direction and magnitude of the fold. In [(water)Co{(DO)(DOH)bn}(ethyl)]$^+$ the fold is downward toward the small OH_2 ligand; replacing water with imidazole causes the equatorial ring to bend upward toward the organic ligand (in the [(imidazole)Co{(DO)(DOH)bn}(ethyl)]$^+$ and [(imidazole)Co{(DO)(DOH) bn}(propyl)]$^+$ complexes).

Since the crystal structure of [(imidazole)Co{(DO)(DOH)bn}(ethyl)]$^+$ has two molecules in the asymmetric unit,[27] we had the opportunity to observe the variable geometry in these complexes. In molecule 1, Co, N(2), N(3), C(5) and C(8) are coplanar within $\pm$0.04(4) Å. In molecule 2 the seven-membered chelate ring is twisted: C(5) is above the equatorial Co–N_4 plane by 0.45(3) Å and C(8) is below the plane by 0.28(3) Å. A similar twisted conformation was seen in a Co(III) methyl tropocoronand that has two seven-membered rings.[28] In all **II** complexes, C(6) and C(7) bend down toward the ligand L (Figure 2); the steric effect of the four-carbon bridge forces the imidazole ligand to lie in a plane that bisects the two diimine chelate rings.

TABLE 1 Wooster Analogues $[LCo\{(DO)(DOH)bn\}R]^+$ Compared with B_{12} Coenzymes. Fold angle is defined as the angle between planes N(1)–C(2)–C(3)–N(2) and N(3)–C(10)–C(11)–N(4) in Wooster analogues. The angle is positive when the folding is upward toward the R group.

R	L	Co-R (Å)	Co-L (Å)	Co-C_α-C_β angle (°)	Fold Angle α (°)	Reference
Et	H_2O	2.012(6)	2.119(3)	119.3(5)	− 6.0	26
Et	im	2.05(2)	2.06(2)	118(2)	4(1)	27
Et	im	2.01(2)	2.05(2)	120(1)	9(1)	27
Pr	im	2.019(7)	2.071(6)	120.9(6)	9.8(5)	29
MeCbl	DMB	1.99(2)	2.19(2)		15.8[a]	5
AdoCbl	DMB	2.023,[b] 2.04[c]	2.214,[b] 2.24[c]	121[b]	13.3[d]	21, 22

[a]As defined in ref 5.
[b]Neutron model at 15 K, ref 22.
[c]Neutron model at 297 K, ref 21.
[d]Ref 19.

Several generalizations can be drawn from the structures of these organocobalt complexes (Table 1). First, the Co–C bond distance is smallest for the methyl group; it increases slightly with the steric bulk of the organic group, but does not seem to be affected by the nature of L. Second, the Co–C_α–C_β angle is widened from the expected tetrahedral angle to about 120°. Third, the Co–O (water) distance is significantly longer than the Co–N_{ax} (im) distance, indicating that the Co–L distance increases for a weaker σ donor.[24] Fourth, the equatorial ligand folds upward, toward the R ligand, when L = im. The extent of folding is variable: there is no regular relationship between R, L, and the fold angle.

The structures of **II** with L = im and R = ethyl[27] or propyl[29] are of particular interest because they can be regarded as simplified models for coenzyme B_{12} in the Class I AdoCbl enzymes, where the imidazole side chain of a histidine is coordinated to the Co. There are a number of structural similarities between these complexes and AdoCbl. The flexible equatorial ligand in **II**, with imino N donor atoms and "built-in" fold angle, mimics the corrin ring. The charge (−1) on the equatorial ligand is the same as the corrin ring, so **II** complexes provide good models for the electrochemical properties of coenzyme B_{12}.[30] The Co–C bond distances are similar to AdoCbl, as are the widened Co–C_α–C_β bond angles (Table 1). The counterions in the crystal (either ClO_4^- or PF_6^-) accept weak hydrogen bonds from the N–H of the imidazole ligand. This is similar to the situation in methyl transferases[14] and glutamate mutase,[8] where the imidazole ligand donates a N–H hydrogen bond to a carboxylate group of the protein.

The exact mechanism of Co–C bond cleavage in AdoCbl is still unknown.[31] Crystallographic studies comparing enzyme-cofactor and enzyme-cofactor-substrate complexes have shown that there is a significant structural change in the enzyme when the substrate binds and that the Co–C bond homolysis occurs during this step.[8,9] It has been proposed that this protein conformational change forces the 5′-deoxyadenosyl group away from the Co by steric clashes, thus breaking the Co–C bond.[8,9] The crystal structures of the enzymes have refined and directed our thinking about mechanism. However, we do not know the details of the process by which the substrate-binding energy facilitates the Co–C bond homolysis.

SEMI-EMPIRICAL STUDIES OF B_{12} MODEL COMPLEXES

We investigated whether a protein-initiated movement of the imidazole ligand toward the Co might lengthen and weaken the Co–C bond.[29] Since the Co(II)–N_{ax} distance in B_{12r}, the reduced form of coenzyme B_{12} with no R ligand, is shorter than the Co(III)–N_{ax} distance in AdoCbl,[23] it seemed reasonable to model the changes in Co–R as Co–N_{ax} decreases. Many other researchers have suggested that the *trans* effect or the *trans* influence might assist or trigger Co–C bond cleavage.[16,32] (Increasing the ease of substitution of the *trans* ligand is called the *trans* effect; this is a kinetic effect. Weakening the bond to the *trans* ligand in the ground state is called the *trans* influence; this is a thermodynamic effect.[33])

Our semi-empirical molecular orbital calculations employed the PM3(tm) model.[34] This model includes d-type atomic orbitals and correctly predicts the geometry of transition metal and organometallic compounds, although energies are not accurate.[35] At the outset, we verified that the semi-empirical calculations would accurately reproduce the known crystal structure (Figure 3). Initial molecular mechanics minimization gave a rather poor model (Figure 3a). After semi-empirical optimization, most bond distances and bond angles agreed within 0.1 Å and 10° (Figure 3b). The few exceptions occurred in flexible areas of the structure—the H bond that closes the macrocyclic ring and the butane bridge, where there were larger deviations between crystal structure and semi-empirical model. The Co–C bond length agreed within 0.03 Å. Models were then built with three equatorial ligands: (DO)(DOH)bn (Wooster analogues), (DO)(DOH)pn (Costa complexes,[36] where pn signifies a propane bridge), or bis(dimethylglyoximato) (cobaloximes). The axial ligands L were imidazole or water, with a variety of alkyl ligands R.

The data plotted in Figure 4 show clearly that the optimized Co–C bond lengths at various constrained Co–L distances increased as the L ligand was brought closer and closer to the Co atom. For the most part, these model compounds behave very similarly, and the data for all three series of complexes coincide closely to the curve for $[LCo\{(DO)(DOH)bn\}(ethyl)]^+$

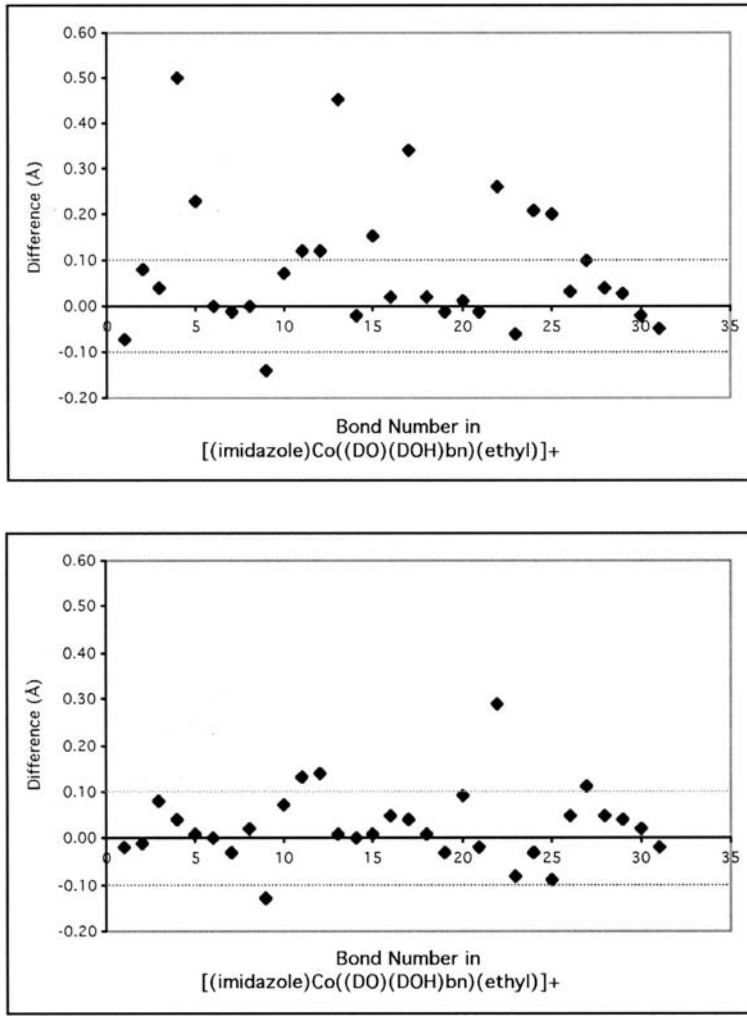


FIGURE 3 Verification of semi-empirical modeling of [(imidazole)Co{(DO)(DOH)bn}(ethyl)]$^{+}$: (a) Difference between crystallographic and molecular mechanics energy-minimized bond lengths; (b) Difference between crystallographic and semi-empirical optimized bond lengths. The largest deviation after semi-empirical modeling is the H(1)···O(1) H bond length. Other deviations ≥ 0.1 Å are three C–C distances in the flexible butane bridge of the equatorial ligand and one N–C distance in the imidazole ring.

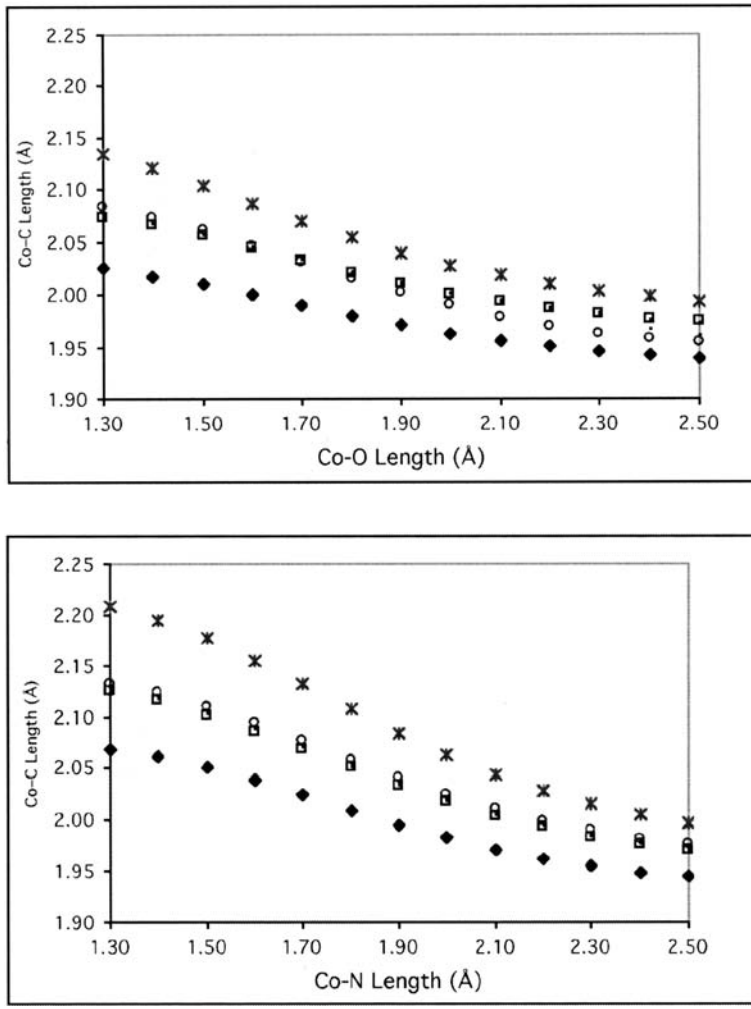


FIGURE 4 Modeling the *trans* influence. Co–C bond distances as a function of constrained Co–L distances in (DO)(DOH)bn (**II**), (CO)(DOH)pn (Costa), and $(DH)_2$ (cobaloxime) B_{12} model complexes with methyl, ethyl, propyl, butyl, 1-methylpropyl and 2-methylpropyl ligands: (a) Constrained Co–OH_2 distances; (b) Constrained Co–N(imidazole) distances. The symbol * represents $[LCo\{(DO)(DOH)bn\}(1\text{-methylpropyl})]^+$; ○, $[LCo\{(DO)(DOH)bn\}(2\text{-methylpropyl})]^+$; □, $[LCo\{(DO)(DOH)bn\}(propyl)]^+$; ◆, $[LCo\{(DO)(DOH)bn\}(methyl)]^+$; —, all other complexes.

(the central curves in Figure 4a and 4b). The 2-methylpropyl complexes that branch at C_β (as does 5′-deoxyadenosyl) also followed the central trend. Only the 1-methylpropyl ligand, in which the alkyl ligand branches at C_α, has significantly longer Co–C distances than 2-methylpropyl at every Co–L distance. The Co-methyl distances are consistently shorter than all the others and change the least. This is consistent with the fact that MeCbl has a different mechanism from AdoCbl.

The magnitude of the *trans* influence in these models correlates with the σ donor power of L. When the Co–OH_2 distance is shortened by 1.2 Å, the maximum extension in the Co–C distance for 1-methylpropyl is 0.14 Å (Figure 4a). Water is a relatively weak σ donor.[24] For L = im, the Co–C distance lengthens to a significantly larger extent, 0.21 Å (Figure 4b). Imidazole is a stronger base, more nucleophilic than DMB,[23] and has a considerably stronger *trans* influence than water in our modeling experiments (Figure 4b).

The sigmoidal shape of the plots in Figure 4 shows that the Co–C bond length is less variable than the Co–L distance. When the Co–N_{ax} distance decreased by 1.2 Å for imidazole models in Figure 4b, the Co–C distance lengthened by 0.21 Å at most (for 1-methylpropyl). When the Co–N_{ax} distances are long, as in a "base off" five-coordinate complex, the Co–C distances vary by only 0.06 Å (1.94 Å for methyl to 2.00 Å for 1-methylpropyl). As the Co–L distance shortens, steric effects of the R ligand are more significant, and the range of distances increases to 0.14 Å (2.07 Å for methyl to 2.21 Å for 1-methylpropyl). The changes in the equatorial ring folding were not uniform; there was no obvious pattern as the Co–R distance decreased. These results do not support a *trans* steric influence, in which corrin ring folding causes the alkyl group to move away from the Co atom. The results do indicate a *trans* electronic influence: shortening the Co–L bond increases electron density on Co and lengthens the Co–C bond by as much as 0.21 Å. Such a substantial increase in length would be expected to weaken the bond considerably.

OTHER RECENT SOLUTION AND MODELING STUDIES

Recent solution studies have investigated the effect of L on the Co–C bond strength in coenzyme B_{12} and its derivatives. While resonance Raman stretching frequencies for Co–C bonds decrease in the order MeCbl > EtCbl > AdoCbl just as do the bond dissociation energies, the force constant is unaffected when the DMB ligand is displaced ("base off").[37,38] In contrast, Sirovatka and Finke[39] found that the rate of Co–C bond thermolysis in adenosylcobinamides (lacking DMB) increased with concentration of added bulky base, showing that the bulky base was involved in the rate-determining cleavage of the Co–C bond. Since the base-induced rate increase was only

slight, they concluded that axial-base effects dominate in the transition state rather than the ground state.

Finke et al.[40] used molecular mechanics (Universal Force Field) to investigate whether short Co–L distances are implicated in the mechanism for Co–C bond cleavage. They found that as the Co–N bond length was decreased, the fold angle of the corrin ring, the Co–C bond length, and the $Co–C_{\alpha}–C_{\beta}$ angle increased. According to their calculations, an increase of at least 0.1 Å in the length of the Co–C bond is necessary to account for the enzymatic rate acceleration of homolysis; the changes they found from their molecular mechanics calculations were $\leq 1/3$ the required magnitude.

Brown and Marques[13] modeled AdoCbl by molecular mechanics (parameterized for cobalt corrinoids) and semi-empirical calculations (ZINDO/1 model) and reached the same conclusions with respect to the ground state effect of decreasing the Co–L distance. Since the elongation of the Co–C bond was only 0.035 Å, they concluded that the ground state effect was not large enough to account for the rate acceleration in the enzymatic reactions. However, they found that decreasing the Co–L bond in the transition state did provide energy stabilization comparable to the $\Delta\Delta G^{\ddagger}$ in enzymes such as ribonucleoside reductase or glutamate reductase. They interpreted these results as support for "transition state mechanochemical triggering" of Co–C bond homolysis in AdoCbl.

Several groups have applied density functional theory (DFT) calculations to the question of whether or not a *trans* influence exists in cobalamins and the extent to which it may be part of the mechanism of Co–C bond homolysis. Because of computational limitations, these DFT computations employed an abbreviated corrin model without amide side chains or nucleotide tail. A study[41] with DMB and either methyl or methyl tetrahydrofuran (as a model for the 5′-deoxyadenosyl ligand) gave evidence for a small *trans* influence when $Co–N_{ax}$ bond distances were frozen. However, the calculated bond dissociation energy changed very little when the axial base distance was either shortened or lengthened. Another DFT study[42] indicated that HOMO energy is higher in AdoCbl than in MeCbl, consistent with the two different reactions these cofactors undergo. Most recently, Jensen and Ryde[43] found that the $Co–N_{ax}$ force constant in cobalamins is relatively weak; the bond can be stretched or compressed by the protein. However, there was no support for the mechanochemical trigger mechanism (*trans* steric effect), since the corrin ring structure changed little when the $Co–N_{ax}$ distance was decreased. They found that compressing the $Co–N_{ax}$ distance led to a very slight (0.03 Å) increase in the Co–C distance, a trend that they attributed to a *trans* inductive effect. Because the imidazole donates a N–H hydrogen bond to a carboxylate in some proteins,[8,14] imidazolate may be a better model than imidazole in these enzymes. Decreasing the Co–N(imidazolate) distance lengthened the Co–C bond distance by 0.07 Å. The resultant weakening of the Co–N(imidazolate) bond could contribute up to 20% of the activation energy needed to homolyze the Co–C bond.

CONCLUSIONS

The two B_{12} cofactors feature an unusual metal-carbon bond that breaks either homolytically or heterolytically, depending upon the carbon ligand. Even though the cobalamins have been intensively studied by scientists for more than 50 years, the exact mechanism by which these intriguing cofactors work is not yet completely understood. The recent protein crystal structures are enormously informative in that they show the protein and cofactor before and after Co–C bond cleavage. We now know that in some enzymes the imidazole ligand is substituted for the dimethylbenzimidazole group before substrate binds. In the structures with both AdoCbl and substrate, a side chain of the protein occupies part of the space vacated by the 5′-deoxyadenosyl group. It appears that when the substrate binds, protein movement at the upper side of the corrin ring may push the 5′-deoxyadenosyl group off the cobalt.[8] Modeling and solution studies described in this review have investigated whether the protein-guided compression of the Co–N_{ax} bond might lengthen and weaken the Co–C bond. We suggest that in Class I AdoCbl enzymes, the process of cleavage involves several factors: protein-induced movement of the imidazole (or imidazolate) ligand that lengthens and weakens the Co–C bond, as well as steric clashes between the protein and the 5′-deoxyadenosyl ligand that further widen the Co–C_α–C_β bond angle. Both of these factors would destabilize the Co–C bond in AdoCbl but would be less effective in MeCbl. This conclusion is consistent with the fact that the two B_{12} cofactors react by different mechanisms. We do not yet know what other structural changes accompany the entire cyclic process of enzyme activation and re-activation. There are likely to be many subtle ways in which the protein controls and directs the mechanism of the B_{12}-dependent enzymes and many more surprises for researchers studying these fascinating B_{12} molecules.

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