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Published in:
Journal of molecular structure. Theochem

DOI:
10.1016/S0166-1280(02)00049-0

2002

Document Version:
Peer reviewed version (aka post-print)

The axial *N*-base has minor influence on Co-C bond cleavage in cobalamins

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2017-04-09

Abstract

We have investigated the properties of cobalamin complexes with imidazolate using the density functional B3LYP method. In particular, we have compared imidazolate (Imm) with imidazole and 5,6-dimethylbenzimidazole (DMB), and studied how constraints in the axial Co-N bond length may affect the strength of the Co-C bond. The results show that the optimum Co-N_{Imm} bond is ~0.2 Å shorter than that of imidazole. There is no indication from crystal structures that the histidine ligand would be deprotonated in the enzymes. However, it is likely that it attains some imidazolate character through its hydrogen bond to a conserved aspartate residue. The Co-N bond with imidazolate is three times more rigid than that with imidazole or DMB, but twice as flexible as the Co-C bond. Constraints in the Co-N_{Imm} bond length give rise to a larger change in the corrin conformation than imidazole, but smaller than for DMB. The resulting effect for the Co-C bond dissociation energy is larger for imidazolate than for imidazole or DMB. However, even the largest reasonable distortion can only enhance catalysis by 15 kJ/mole. Therefore, we conclude that, irrespectively of the nature of the *N*-base, constraints in the axial Co-N bond lengths cannot be the main reason for the catalytic power of cobalamin enzymes.

Key words: Coenzyme B_{12} Density functional theory Mechanochemical trigger mechanism Butterfly effect Cobalamin Ligand triad

1. Introduction

The chemistry of cobalamins has attracted much interest during the last 40 years, especially the unusual organometallic Co-C bond, which is broken during catalysis. The general structure of cobalamin is shown in Figure 1. Two of the rings are directly connected, and the outer carbon atoms of the corrin ring are sp^3 hybridised. These structural features render the corrin system different from porphyrins [1]. In some cobalamin enzymes, the 5,6-dimethylbenzimidazole (DMB) group at the end of the long nucleotide tail coordinates to cobalt also in the protein [2,3], whereas in many other enzymes, the imidazole ring of a histidine residue replaces DMB as the -side ligand [4-6]. The two native cobalamin coenzymes are methylcobalamin (MeCbl) and 5'-deoxyadenosylcobalamin (AdoCbl). A well-established dichotomy has been observed for these two coenzymes: MeCbl acts as an methylation reagent by a heterolytic cleavage of the Co-C bond, whereas AdoCbl acts as a radical generator through a homolytic cleavage of the Co-C bond [7].

Vast experimental effort has been directed towards the understanding of how the Co-C bond is labilised and how the reactivity of the two coenzymes differs. Recently, it has been shown that the homolysis-heterolysis preferences of the isolated coenzymes actually differ [8-11]. Quantum chemical calculations have suggested that this is reflected by differing frontier-orbital energies of AdoCbl and MeCbl models [12]. These results indicate that the difference is at least partly a feature of the ground states of the isolated coenzymes rather than an effect of the enzyme.

Cobaloximes, i.e. *R*-*L*-bis(DMG)cobalt complexes (where DMG = dimethyl glyoximate), have been used extensively as cobalamin model systems, both in experiments [13-25] and theory [26-28]. Much of this research has focussed on the flexibility of the bis(DMG) ligand, indirectly interpolating this flexibility to corrins. However, the corrin ring is substantially more rigid than bis(DMG), as semiempirical studies on both kinds of model complexes have shown [29]. In addition, the cobaloxime charge and electron density on cobalt differ from those of corrins [30], mainly because bis(DMG) is a dianionic ligand, whereas corrin is mono-anionic.

This focus on the flexibility of the corrin ring has led to the proposal of the mechanochemical trigger mechanism [28,31-44]. This often cited hypothesis explains the labilisation of the Co-C bond by means of trans steric effects, i.e. that the *N*-base may induce an upwards folding (butterfly bending) of the corrin ring, causing the Co-C bond to elongate and hence to weaken. The strain energy of the corrin ring would favour a square-pyramidal Co^{II} homolysis intermediate. However, in the case of heterolysis, such a mechanism is a priori unlikely, because the formation of the experimentally observed square-planar Co^{I} intermediate [45,46] would require the Co-N distance to increase rather than decrease.

Two recent theoretical papers have studied the importance of the mechanochemical trigger mechanism for the catalysis of cobalamin enzymes. Finke et al. suggested on the basis of classical force-field (UFF) methods that a mechanochemical trigger may contribute with at most 1/3 of the enzymatic enhancement of the homolysis rate [47]. This was confirmed in calculations using another classical force field (MM2), which showed that mechanochemical trigger could account for no more than 17 kJ/mol of the observed catalytic enhancement (0 kJ/mol) [44]. However, using further classical and semiempirical calculations, they suggest that a compression of the Co-N_{ax} bond *in the transition state* may account for all of the typical 10^{10}-10^{12} rate enhancement.

Recently, several groups have started to use density functional theory (DFT) to acquire insight in the reactivity of cobalamins [12,48-52]. For example, it has been shown that if the Co-C bond length is increased (as an effect of a change in the -side ligand), the trans Co-N_{ax} bond length also increases (i.e. an inverse trans effect) [12,48]. Moreover, it has been shown that the Co-C bond dissociation energy is linearly related to the Co-C bond length and that it is little affected by variations in the Co-N_{ax} bond length or the type of *N*-base [50,52].

All these studies have concentrated on the DMB or neutral imidazole form of the coenzyme. However, the imidazole side-chain of the histidine ligand present in some proteins normally forms a hydrogen bond to a conserved carboxylate group [4,5]. It has

been shown that mutation of the latter residue leads to a 15-1000 times lower catalytic activity in glutamate mutase and methionine synthase [53,54]. The other carboxylate oxygen of this group typically forms a hydrogen bond to a serine or lysine residue [4]. Such a ligand triad has been suggested to be important for the mechanism of many cobalamin-dependent enzymes and could be one reason why many B_{12} enzymes replace the DMB group by a histidine ligand [7]. In particular, it could induce the formation of an imidazolate ion during the reaction of the enzymes. Similar metal-histidine-carboxylate patterns are found in many other proteins and has been shown to be important for the understanding of the reactivity and other properties of the metal ion [55-57].

Therefore, we study in this paper the properties of cobalamins involving a deprotonated imidazolate group. We investigate how constraints in the axial Co-N_{ax} bond length may change the conformation of the corrin ring and the reactivity of the coenzyme. We also compare the properties of imidazolate cobalamins with those of complexes with imidazole and DMB. Thereby, we improve the earlier calculations with better methods or larger basis sets and we get the opportunity to discuss various aspects of trans effects in cobalamins. Our results indicate that the histidine ligand is not deprotonated in the ground state of any structurally characterised protein, but that the hydrogen bond to the carboxylate increases the basicity of the ligand. Moreover, trans effects, even those involving imidazolate, are small in terms of energy and geometry, and therefore has a small influence on the reaction of cobalamins.

2. Methods

2.1.Model systems

The geometry of nine corrin models has been fully optimised. In all cases, we included the entire corrin ring (Cor) in the models, but the amide side chains as well as the nucleotide tail were replaced by hydrogen atoms (see Figure 1). Two oxidation states of cobalt were examined; the closed-shell Co^{III} state and the doublet Co^{II} state. All complexes were considered to be in the low-spin state, in accordance with experiment

[1]. As the -side ligand, we used either 5,6-dimethylbenzimidazole (DMB), imidazole (Im), or imidazolate (Imm). Three square-pyramidal Co^{II} homolysis intermediates were optimised: $Co^{II}CorIm^{+}$ $Co^{II}CorImm$ and $Co^{II}CorDMB^{+}$. Six octahedral Co^{III} models were studied, three methyl (Me) cobalamin models and three AdoCbl models, both with either DMB, Im, or Imm as the -side ligand. We used 5'-deoxyribosyl (Rib) as a model of 5'-deoxyadenosyl because in vacuum calculations, adenosine interactions with the corrin ring may produce an artificial distortion of the ring. In protein crystals, the adenine moiety is at least 4 Å from the corrin ring [5,58]. Therefore, realistic simulations of the full adenosine ligand must be performed with a much larger model of the active site, e.g. in combined quantum mechanical and molecular mechanical calculations.

For all optimised structures we performed a relaxed scan of the Co-N_{ax} bond length by increasing or decreasing the optimal Co-N_{ax} bond length by 0.15 or 0.30 Å, and optimising the remainder of the structure. Similar scans were also performed for the Co-C bond in $CoCorImMe^{+}$, $CoCorImmMe^{+}$, and $CoCorImRib^{+}$, but this stiffer bond length was only changed by 0.05 or 0.1 Å.

2.2.Computational details

All calculations were performed with the Becke three-parameter hybrid functional, using the local spin-density approximation correlation functional of Vosko-Wilk-Nusair and the non-local Lee-Yang-Parr correlation functionals (B3LYP) [59-63]. B3LYP is widely recognised as one of the most accurate density functional methods, in general terms, for structures, energies, and frequencies, and is highly successful in transition metal chemistry [64-68]. B3LYP incorporates correlation at a significantly lower cost in terms of cpu-time than perturbation theory, configuration interaction, and coupled-cluster techniques. It is only slightly slower than analogous Hartree-Fock calculations, but significantly more accurate.

The calculations were carried out with the Turbomole software, version 5.3 [69]. The basis sets used for geometry optimisation and potential energy curves were 6-

31G(d) for all atoms except cobalt, which was described by the double- basis set of Schäfer *et al.* [70], augmented with one *f* (exponent 1.62), one *d* (0.1357), and two *p* functions (0.141308 and 0.043402) with the contraction scheme (14s11p6d1f) / [8s7p4d1f] (called DZpdf below). This basis set assigns one set of polarisation functions to all non-hydrogen atoms. Only the pure five *d* and seven *f*-type functions were used.

Accurate energies for the optimised structures were calculated with the triple- 11+G(2d,2p) basis set, which includes diffuse functions on heavy atoms and two polarisation functions on all atoms. For cobalt, we used the DZpdf basis set with an additional *s* function (0.0145941), and with two *f* functions (exponents 2.8 and 0.8) replacing that of DZpdf. We applied the default (m3) grid size of Turbomole [71], and all optimisations were carried out in redundant internal coordinates [72]. Unrestricted calculations were performed for the open-shell systems. We made use of default convergence criteria, which imply self-consistency down to 10^{-6} Hartree (2.6 J/mol) for the energy and 10^{-3} a.u. (0.053 pm or 0.057°) for the internal degrees of freedom.

3. Results and Discussion

3.1 Optimised structures

The structures of all optimised complexes are shown in Figure 1 and the bond lengths to the cobalt ion are collected in Table 1. The equatorial Co-N_{eq} distances range from 1.89 to 1.95 Å. This is close to what is found in crystallographic studies of MeCbl, AdoCbl, and Co(II)Cbl, 1.81-1.98 Å [30,73-76]. The Co-N_{eq} distances do not vary much with the nature of the complex, neither in experiments nor in our calculations (c.f. Table 1) [12]. Therefore, they are not further discussed in this paper.

The axial Co-C bond lengths vary somewhat more (1.96-2.02 Å). The bond length in $CoCorDMBMe^+$ (1.96 Å) agrees well with the experimentally determined distance in MeCbl: 1.98-1.99 Å with X-ray crystallography [30,75] and 2.00±0.03 Å with extended X-ray absorption fine structure (EXAFS) measurements [46]. The bond length is similar in the corresponding imidazole complex, whereas imidazolate gives

rise to a 0.03 Å longer bond. Similar trends are observed if the -side ligand is changed to Rib, but the Co-C bond of Rib is 0.03 Å longer than the corresponding methyl bond length. Exactly the same trend is found in crystal structures of AdoCbl and MeCbl [30,75].

For the axial Co-N_{ax} bond length, variations are even larger, ranging from 2.08 to 2.39 Å in the various complexes. The bond is longest (2.32-2.39 Å) for DMB, whereas imidazole gives 0.07-0.11 Å shorter bonds. This reflects the higher basicity of imidazole and the larger steric demands of DMB. A similar difference has been obtained in other calculations [48], and a slightly smaller shortening (0.04 Å) has been observed experimentally when DMB is replaced by imidazole in cyanocobalamin [77]. Imidazolate gives rise to even shorter Co-N_{ax} bond lengths by 0.12-0.20 Å, more for Co^{III} than for Co^{II}. Ribosyl gives a 0.03-0.06 Å longer Co-N_{ax} bond than methyl in the imidazole and DMB complexes, but there is hardly any difference in the Co-N_{ax} bond lengths in the imidazolate complex.

3.2 The Co-N_{ax} potential surface

We have seen that the optimised Co-C and Co-N_{eq} bond lengths are close to those obtained experimentally. However, for the Co-N_{ax} bond length, there is an appreciable discrepancy: The experimental Co-N_{ax} distance in MbCbl is 2.16-2.19 Å (X-ray) and 2.20 ±0.03 Å (EXAFS) [30,46,75], whereas we obtain 2.32 Å. Likewise, the experimental Co-N_{ax} distance in AdoCbl is 2.19-2.24 Å, compared to our 2.39 Å [73,74]. These differences are larger than the normal 0.04-0.07 Å overestimation of metal-nitrogen bond distances observed in other systems studied with the same method [78-80], but it is in line with what has been found in other B3LYP studies of corrins [12,49,52].

In order to understand this difference, we calculated the potential energy surface of the Co-N_{ax} bond in the various complexes by constraining this bond at various distances and reoptimising the rest of the structure. The result in Figure 3 shows that for imidazole and DMB, the potential is extremely flat. The force constant of this bond is

approximately 100 kJ/mole/Å^2, which is 5-6 times lower than for the opposite Co-C bond (see below). This is in line with earlier theoretical results [52] and with the low frequency of the Co-N_{Im} vibration (128 cm^{-1}) calculated by Kozlowski and coworkers [82]. In particular, Figure 3 shows that the difference in the Co-N_{ax} bond distance between theory and experiments corresponds to only 2-3 kJ/mole in energy terms. This explains the discrepancy and shows that this bond length can easily be modulated by interactions with the surroundings, e.g. in a protein or in crystals. Moreover, it provides a direct quantification of the predicted "substantial energy cost" of Co-N_{ax} bond compression [83].

However, deprotonation of imidazole renders the Co-N_{ax} bond less flexible, as could be expected from an interaction between two oppositely charged groups. Figure 3 shows that, independent of the -side ligand, the force constant of this bond is ~300 kJ/mole/Å^2, i.e. three times larger than for imidazole and of a strength similar to normal metal-imidazole bonds [84], but still half as strong as the Co-C bond. This indicates that it is appreciably harder (costs more energy) to modify the Co-N_{ax} bond length of imidazolate, but on the other hand, more energy can also be stored in this bond.

3.3.*The Co-N_{ax} bond in protein structures*

One interesting questions is whether there is any experimental indication that the histidine ligand is deprotonated in the protein. Since imidazolate is predicted to have a 17-pm shorter Co-N_{ax} distance, it might perhaps be identified in crystal structure of proteins. Therefore, we have studied the crystal structures of five different cobalamin-binding proteins, viz. methionine synthase, methylmalonyl coenzyme-A mutase, glutamate mutase, diol dehydratase, and ATP:corrinoid adenosyltransferase. In the former three proteins, histidine is the -side ligand, whereas in the latter two, DMB is bound to cobalt also in the protein. The latter enzymes were included to get a feeling of the uncertainties in the distances and to make the investigation complete. The cobalt-ligand distances in all available crystal structures of these proteins are collected in Table 2. From this compilation, it can be seen that there is a large spread in all distances, even

in the Co-N_{eq} distances (1.56-2.08 Å). This is probably an effect of the low resolution of the structures (2-3 Å).

The interpretation of several X-ray structures is complicated by the fact that they are partly or fully reduced to Co^{II}. A -side carbon ligand is only present in six structures, and normally only with a partial occupancy and a considerable spread in the Co-C distance (1.91-2.16 Å). In five structures, which are predominantly in the Co^{III} state, the Co-N_{ax} distance is rather short, 2.17-2.22 Å (averages of the two subunits found in most crystal structures). This is very similar to the experimental bond length in in the free coenzyme, e.g. 2.16-2.20 Å for MeCbl [30,75]. Thus, there is no indication that the histidine ligand in the proteins is deprotonated. According to our calculations, such a Co-N_{ax} bond length should be around 2.08 Å, and considering that B3LYP typically overestimates metal-ligand bond lengths, it should probably be at least 0.04 Å shorter. None of the Co-N_{ax} bond lengths in the proteins is shorter than 2.11 Å.

On the contrary, in most of the structures (which are fully or partly reduced) the bond is appreciably longer, 2.39-2.72 Å (averages). Only two structures, which are suggested to be a mixture of Co^{II} and Co^{III}, have an intermediate distance (2.28-2.34 Å). Thus, Co^{III} complexes, with or without a -side ligand, seem to give a normal Co-N_{ax} bond length, whereas the five-coordinate Co^{II} complexes give rise to a long Co-N_{ax} bond length in the proteins [5,58,85-88].

Interestingly, this is not in accordance with experimental data of the free coenzyme: the crystal structure of cob(II)alamin shows a Co-N_{ax} bond length of 2.13 Å [89], i.e. 0.03 Å *shorter* than for MeCbl and ~0.10 Å shorter than for AdoCbl [30,73-75]. In our calculations, both Co^{II}CorDMB$^+$ and Co^{II}CorIm$^+$ have the same Co-N_{ax} bond length as in the corresponding Co^{III}CorMe complexes. Likewise, Figure 3 shows that the Co^{II}-N_{ax} bond has the same soft potential energy surface as the Co^{III}-N_{ax} bond. In fact, the curves are almost identical. From the curves, it can be seen that the Co^{II}-N_{ax} bond lengths observed in crystal structures of both free and protein-bound cobalamins can be obtained at a cost of less than 5 kJ/mole, again illustrating the flexibility of this bond.

3.4. *Importance of a long Co-N_{ax} bond in proteins*

The observation of a long Co-N_{ax} bond (~2.5 Å) in many protein structures (cf. Table 2) has led to the suggestion that the enzymes may promote homolytic cleavage of the Co-C bond by elongating the trans axial bond [5]. More precisely, such an elongation would stabilise the Co^{II} intermediate relative to Co^{III} [5,10] because low-spin Co^{II} has a 0.12-Å larger ionic radius than low-spin Co^{III} [90]. Thus, the protein might maintain a constant and long Co-N_{ax} bond that would be strained for Co^{III}, but close to ideal for Co^{II} [10].

However, there are several lines of evidence against this suggestion. First, crystal structures of the free coenzyme show that the ideal Co-N_{ax} bond length of the Co^{II} form is not longer than that of the Co^{III} form, but rather slightly *shorter*. The reason for this is the change in the coordination number from six to five. In the five-coordinate Co^{II} complex, the cobalt ion is ~0.1 Å below the the corrin plane towards the DMB ligand (both in experiments and our calculations), giving rise to a shortened Co-N_{ax} bond length [76].

Second, our results in Figure 3 clearly show that the Co^{II} and Co^{III} complexes have almost identical Co-N_{ax} potential curves. Therefore, the Co^{II} and Co^{III} complexes would be destabilised to a similar extent by a certain constraint in the Co-N_{ax} bond length, and the Co^{II} species would be *more* destabilised if its optimal bond length is shorter than that of the Co^{III} species. Thus, our calculations give no support to the suggestion that the axial *N*-base effect should be greater for Co^{II} than for Co^{III} [10].

Third, the potential curves show that the destabilisation energy of constraints in the Co-N_{ax} bond is small, less than 6 kJ/mole. Such an energy is small in comparison with the strength of the Co-C bond (109-155 kJ/mole) [50] and the suggested catalytic enhancement of the reaction rate caused by the protein (10-13 orders of magnitude or ~70 kJ/mole) [76,91,92].

Finally, it should be noted that a recent EPR study indicates that the Co^{II}-N_{ax} distance is not particularly long in any of five studied proteins [93]. Similarly, EXAFS

measurements have indicated that the Co^{III}-N_{ax} bond is not lengthened in glutamate mutase and 2-methyleneglutarate mutase [94]. Hence, there is both experimental and theoretical evidence against the ideal Co-N_{ax} bond hypothesis.

3.5.The importance of a mechanochemical trigger mechanism

The mechanochemical trigger mechanism is an old, but still viable hypothesis, which explains how proteins may promote the cleavage of the corrin Co-C bond [31,32,44]. According to this hypothesis, the protein compresses the Co-N_{ax} bond, leading to an upward folding of the corrin ring, which is expected to elongate and weaken the Co-C bond by Van der Waals repulsion between the corrin ring and the -side ligand.

The upward folding of the corrin ring is usually measured by the so-called corrin fold angle. It is defined as the angle between the two average planes formed by the seven inner atoms in the corrin ring on both sides of a line from the missing methine link to the opposite methine atom [1]. We have measured this angle in all the optimised complexes (Table 3). The angle is 2-4° lower for imidazole than for the DMB complexes. This is expected, since DMB is a more bulky group. The fold angle is 1 lower for ribosyl than for the methyl group, which probably is caused by the longer Co-C and Co-N_{ax} bond lengths. The angle is approximately the same for Co^{II} and the corresponding Co^{III}CorMe complex. All these trends are in excellent agreement with experimental results [30,66-68,95,96]. However, the absolute values of the fold angle are 7-8° smaller in the optimised structures. This is probably an effect of the lack of side chains and the too long Co-N_{ax} bonds in the optimised structures, because the fold angle is inversely related to this bond length [96].

Imidazolate gives 1-3° larger fold angles than imidazole. This is probably an effect of the ~0.17 Å shorter Co-N_{ax} bond lengths, which increase the steric interactions between imidazolate and the corrin ring. However, the fold angles of imidazolate are still 1-2° smaller than those of DMB, which illustrates that the steric bulk of the *N*-base is more important for the corrin distortion than the Co-N_{ax} bond.

Our relaxed scans of the Co-N_{ax} bonds give us an opportunity to directly test the predictions of the mechanochemical trigger mechanism. In Table 3, we show how the fold angle changes with the Co-N_{ax} bond length in the various complexes. The fold angle increases if the Co-N_{ax} bond is shortened, and it decreases when the bond is elongated. This inverse relation has also been observed experimentally (although on complexes with different ligands) [96]. The effect is largest for DMB, intermediate for imidazolate, and smallest for imidazole, exactly as for the actual value of the fold angle. However, for all three bases, the effect is quite limited. For imidazole, the total effect of varying the Co-N_{ax} bond over 0.6 Å is less than 2°, whereas it is 4-6° for DMB. Similar effects have been observed in molecular mechanics simulations [44,47].

The fold angle measures only one mode of deformation of the corrin ring. It is conceivable that other modes may show larger effects in response to variations in the Co-N_{ax} bond length. However, the overlay of the four constrained structures of the $CoCorImMe^+$ and $CoCorDMBMe^+$ complexes in Figure 4 shows that the change in the corrin structure is small for both complexes, although the Co-N_{ax} bond is altered by 0.6 Å. Again, the effect is largest for the DMB ligand, especially in the area where it interacts with the corrin ring.

Thus, the structural effect of constraints in the Co-N_{ax} bond length is limited. However, the *energetic* effects of such constraints are more important for the mechanism of the cobalamin enzymes. These effects can be directly seen from the potential energy surface in Figure 3. Owing to the flat potential surface, the effect is quite small for the uncharged ligands - it costs ~15 kJ/mole to compress the Co-N_{ax} bond by 0.3 Å from the optimum value for DMB, and ~20 kJ/mole for imidazole. This energy includes the cost of changing the fold angle as well as all other geometric changes. This clearly shows that little energy is stored in variations in the fold angle, i.e. that it is a *low-energy mode*.

However, for imidazolate with its more normal potential, there is appreciably more energy stored in the Co-N_{ax} bond: It costs ~50 kJ/mole to compress it by 0.3 Å from the optimum value. Yet, we will see below that the effect on the Co-C bond

strength is still quite small. Moreover, the large energy is not connected with the change in the corrin conformation (folding), as the mechanochemical trigger mechanism predicts, but rather with the compression of the Co-N_{ax} bond.

In conclusion, for all three *N*-bases, the overall change in corrin structure is small even for large deformations of the Co-N_{ax} bond and there is no indication of an important energetic or structural role of the mechanochemical trigger mechanism in the reaction of corrin proteins. This is in accordance with earlier molecular mechanics and density functional calculations [44,47,52]. The conclusion is strengthened by the fact that available crystal structures (c.f. Table 2) provide no indication of a compressed Co-N_{ax} bond, which would be needed to severely affect the energetics of the systems (see Figure 3). On the contrary, they mainly show *elongated* bonds, as was discussed above.

3.6.Trans electronic induction

A variation of the Co-N_{ax} bond length may affect the Co-C bond in at least two different ways: either by trans steric effects as suggested by the mechanochemical trigger mechanism or by trans induction, by which the Co-C bond is elongated upon compression of the Co-N_{ax} bond, owing to the increased electron density on cobalt. The resulting effect on the Co-C bond can in principle be a combined effect, but if steric effects are important, Co-C bond labilisation should be accompanied by a deformation of the corrin ring. Since we have shown that such a deformation is small, we will assign all effects on the Co-C bond to electronic induction in the following.

Compression of the Co-N_{ax} bond leads to an elongation of the Co-C bond length, as is shown in Figure 5. For the total 0.6 Å variation of Co-N_{ax}, the effect on the Co-C bond length is ~0.03 Å for imidazole and DMB. For imidazolate, the effect is about twice as large, 0.06-0.07 Å. This is in accordance with the weak effect observed by Raman studies of the Co-C bond [91,97-101].

This suggests that the Co-C bond is considerably stiffer than the Co-N_{ax} bond. To verify this, we calculated the potential energy curve of the Co-C bond, using the $CoCorImMe^+$, $CoCorImRib^+$, and CoCorImmMe models. The result in Figure 6 confirms that this bond is indeed stiffer; a 0.1-Å variation of this bond costs 4-7

kJ/mole, irrespectively of the axial ligands. This corresponds to a force constant of ~600 kJ/mole/Å^2, i.e. ~6 times larger than for the Co-N_{ax} bond to imidazole and DMB, and even twice as strong as the Co-N_{ax} bond to imidazolate. The size of the force constant is similar to what has been estimated with resonance Raman spectroscopy, e.g. 557 kJ/mole/Å^2 for MeCbl [98] and it is in accordance with a calculated vibrational frequency of 535 cm^{-1} [82].

Most importantly, however, we see that a 0.03-Å variation in this bond corresponds to less than 1 kJ/mole in energy, and even the 0.07-Å variation found for the imidazolate complexes corresponds to an energy of less than 4 kJ/mole. Clearly, such an energy insignificant compared with the dissociation energy of the Co-C bond (109-155 kJ/mole) [50].

3.7.Bond dissociation energies

A more direct test of the importance of Co-N_{ax} bond for the Co-C bond strength is to calculate the (homolytic) bond dissociation energy (BDE) of the Co-C bond as a function of the Co-N_{ax} bond distance. We calculate the BDE as the reaction energy of the dissociation

$$Co^{III}CorImMe^{+} \quad Co^{II}CorIm^{+} + Me \qquad (1)$$

or similarly with other axial ligands. For the complexes with DMB or imidazole, we obtain BDEs of 86-91 kJ/mole with the triple- basis set. This is much lower than the experimental results for MeCbl, 155 12 kJ/mole [102-106]. However, our results are similar to the BDE obtained in other DFT calculations with smaller basis set, 96-117 kJ/mole [50,52], especially as we observe that the BDE decreases as the basis set is increased. The fact that DFT-computed BDEs are in such disagreement with experiment is quite a puzzle, which needs to be investigated further. There are many possible sources of this discrepancy. For example, zero-point energies increase the BDE by 10 kJ/mole [50] and relativistic effects also increase it by 7 kJ/mole (scalar terms only).

However, since we are interested in *relative* energies when the axial ligand or the Co-N_{ax} distance is varied, these absolute errors are less serious.

In Figure 7, we present the calculated BDE of the Co-C bond as a function of the Co-N_{ax} bond length (note that the energies are calculated with the smaller double- basis set and are therefore different from the values cited above), with the assumption that the Co^{III} and Co^{II} complexes are constrained from the equilibrium Co-N_{ax} distance by the same amount (a reasonable assumption, considering the parallel potential energy surfaces in Figure 3 and their similar equilibrium bond lengths in Table 1). We see that for the imidazole and DMB complexes, the Co-N_{ax} bond length has a very small influence on the Co-C BDE; if the Co-N_{ax} bond is displaced by 0.3 Å from its optimum value, the BDE changes by less than 5 kJ/mole. Even more importantly, the BDE *increases*, as the Co-N_{ax} distance is changed (both when it is increased or decreased). Thus, there is no gain of constraining the Co-N_{ax} bond length with a neutral *N*-base. This provides a strong argument against both the mechanochemical trigger mechanism and the ideal bond hypothesis.

For the imidazolate complexes, things are slightly different. The BDE of the CoCorImmMe (108 kJ/mole) and CoCorImmRib (95 kJ/mole) complexes are 9-17 kJ/mole higher than for the imidazole counterparts (with the triple- basis set). This is quite unexpected, because the Co-C bond length in the imidazolate complexes is ~0.03 Å *longer* than in the imidazole complexes. A longer bond length is normally considered to be weaker and an inverse linear relation has been found between the Co-C bond length and BDE [48]. However, the finding is in accordance with the experimental observation of reduced homolysis with an imidazolate -side ligand [11]. It is best understood when discussed in terms of acid constants (next section).

Moreover, it can be seen from Figure 7 that the Co-C BDE changes slightly more when the Co-N_{ax} bond length is varied for imidazolate than for imidazole or DMB, up to 8 kJ/mole for a 0.3-Å variation. More importantly, the shapes of the BDE curves differ: For the uncharged bases, the BDE increases upon variations of the Co-N_{ax} for imidazole, but for imidazolate, the BDE *decreases*. The reason for this is that the Co-N_{ax}

bond of the Co^{II} complex with imidazolate is more flexible than the same bond in the Co^{III} complex (c.f. Figure 3).

Therefore, with imidazolate, there could in principle be a favourable effect of constraints in the Co-N_{ax} bond as suggested by the mechanochemical trigger mechanism or the ideal bond hypothesis. However, Figure 7 shows that the effect would be quite limited, less than 2 kJ/mole if the bond is elongated by 0.3 Å, or up to 8 kJ/mole if the bond is compressed by the same amount. These energies are still only a small fraction of the total Co-C BDE (109-155 kJ/mole) [50] or of the observed catalytic enhancement by the enzymes (0 kJ/mole). Moreover, it must be remembered that there is a large cost of such constraints for the imidazolate ligand; Figure 3 shows that a 0.3-Å elongation of the Co-N_{ax} bond would cost ~15 kJ/mole, whereas the corresponding compression would cost as much as 50 kJ/mole. Such energies are larger than the strain normally observed when a molecule is bound to a protein, up to 10 kJ/mole [107,108].

However, it has been argued that the enzyme could use much of the binding energy of the coenzyme to distort the Co-N_{ax} bond [109]. In fact, it has been observed that coenzyme B_{12}-dependent ribonucleotide reductase binds AdoCbl with an association constant of 2 10^4 M^{-1} (25 kJ/mole), whereas products of Co-C bond cleavage are bound much more tightly (K= 7 10^{16} M^{-2}, or 97 kJ/mole) [44,109]. The difference, 72 kJ/mole could in principle be used to compress the Co-N_{ax} bond (by ~0.35 Å), giving a decrease in the BDE of up to 15 kJ/mole, i.e. ~20% of the observed enzymic rate enhancement. This provides a strict upper limit of the effect of constraints in the Co-N_{ax} bond for cobalamins with an imidazolate ligand.

3.8. Acid constants

The observed changes in the BDEs when imidazole is deprotonated may as well be interpreted as changes in the pK_a of the imidazole ligand in the corrin complexes. The difference in the BDE of the methyl complex with imidazole and imidazolate is the energy of the isodesmic reaction

$$Co^{II}CorImm + Co^{III}CorImMe^{+} \quad Co^{II}CorIm^{+} + Co^{III}CorImmMe \qquad (2)$$

as can be seen by subtracting reaction (1) with imidazolate from the same reaction with imidazole. This reaction energy is -17 kJ/mole with the triple- basis set. However, the reaction can also be interpreted as a comparison of the acid constants of cobalt complexes at two different oxidation states, indicating that in vacuum, the pK_a of $Co^{III}CorImMe^{+}$ is 3 units lower than that of $Co^{II}CorIm^{+}$. This could be anticipated from the larger effective nuclear charge (by 0.35 e) and smaller ion radius (by 0.1 Å) of Co^{III} than Co^{II} [90].

The corresponding reaction for the ribosyl model yields an energy of -9 kJ/mole, showing that the pK_a of methylcorrin is 1.5 units lower than that of ribosylcorrin. This may be explained by the shorter Co-C bond, and therefore larger induction and - overlap of methyl. It is also reflected by a 0.05 *e* higher Mulliken charge of cobalt in the methyl corrin, which makes it interact more favourably with imidazolate. These results show that it is more likely that the histidine ligand of the Co^{III}-coenzyme is deprotonated that of the Co^{II} form. Since our comparison with crystal structures above indicated that the histidine ligand is protonated for the Co^{III} form, this should also be the case for the Co^{II} form.

3.9 The nature of the trans effects

Our results show that trans effects are small in the corrin models. This is in conspicuous contrast to trans effects in the other direction, which have been shown to be strong in series of alkyl ligands and give simultaneous elongation of both axial bonds (the so-called *inverse* trans effect) [12,48]. For example, the Co-N_{ax} bond length to imidazole increases from 2.09 to 2.42 Å as the trans Co-C bond length increases from 1.87 to 2.10 Å in a nearly linear manner [48]. Hence, we see that it is important to distinguish between and trans induction; the former effect is very weak, whereas the latter is stronger and inverse. A comparison of Figure 3 and Figure 6 quantifies this difference: The Co-C bond is ~6 times more rigid than the Co-

N_{ax} bond.

The large trans effect has been attributed to an electrostatic repulsion between N_{ax} and Co: The cobalt atom acquires a higher electron density as the induction ability of the -side ligand is increased [12]. Our results leading to Figure 6 give us the opportunity to elucidate whether the large change in the Co-N_{ax} bond length is caused primarily by the change in the -side ligand or by the change in the Co-C bond length. Figure 8 shows how the Co-N_{ax} bond length changes when the Co-C bond length is varied with the same -side ligand, a methyl group. Interestingly, the effect is quite small; when the Co-C bond length is changed by 0.2 Å, the trans Co-N_{ax} bond length changes by only 0.05 Å. Moreover, it is *no longer inverse*: When the Co-C distance increases, the Co-N_{ax} bond length decreases. This shows that both the large increase of the Co-N_{ax} bond length and the inverse relation observed in earlier studies [12,48] is caused by changes in the ligand rather than by the Co-C bond elongation itself. Hence, the inverse trans effect is not a true trans effect, but rather a substituent effect and hence it is less important for the actual biochemical system, which does not change the ligand during the catalytic cycle.

4. Concluding remarks

In this paper, we have compared the properties of cobalamin complexes with imidazolate to those with imidazole or DMB. In this comparison, we also improve earlier calculations with imidazole or DMB by using better methods or larger basis sets. By comparing the optimum Co-N_{ax} bond lengths of imidazolate with those observed in crystal structures of proteins, we can conclude that it is unlikely that the histidine ligand is deprotonated in any of these structures. Moreover, we have shown that the pK_a of the imidazole ligand is 1.5-3.0 units lower for Co^{III} than for Co^{II} complexes, indicating that it is even more unlikely that the homolytic Co-C bond cleavage product should be deprotonated in the enzymes.

However, this does not mean that studies of imidazolate are uninteresting. It is most likely that the conserved aspartate residue, which forms a hydrogen bond to the

histidine ligand, increases the basicity and give imidazolate character to the ligand. Therefore, the present calculations provide an upper limit of these characteristics. Moreover, it cannot be excluded that the protein stabilises an imidazolate form of some intermediates during the reaction.

The Co-N_{ax} bond of imidazolate is three times more rigid than that of imidazole or DMB. However, it is still twice as flexible than the Co-C bond. Moreover, the Co^{II}-N_{ax} bond is more flexible than the Co^{III}-N_{ax} bond for imidazolate, whereas it is the other way around for the neutral bases. This leads to a qualitative difference in how the BDE varies with constraints in the Co-N_{ax} bond: For imidazolate the BDE decreases if the Co-N_{ax} bond is constrained, whereas for the other two bases, the BDE always increases if such constraints are present.

Our results have several important implications on the mechanism of cobalamin enzymes. There is a controversy in the cobalamin literature whether axial *N*-base trans effects are small or large. Many researchers have interpolated the large trans effects of cobaloximes to corrins [13-16,18-22,24,110]. In this paper, we emphasise an important difference between and trans induction within corrins: trans effects are rather large and caused mainly by electronic induction. On the other hand trans effects are minor, especially for neutral -side ligands.

Our results show that the Co-N_{ax} bond potential energy curve of Co^{III} and Co^{II} complexes with the same axial ligands are parallel and almost identical (Figure 3), and that their ideal bond lengths are similar (Table 1). Therefore, they lend no support to the suggestion [5,10] that a long Co-N_{ax} bond would stabilise Co^{II} relative to Co^{III}. For imidazole and DMB, Figure 7 shows that elongation of the Co-N_{ax} bond would lead to an increase in the BDE, whereas for imidazolate, an elongation could decrease the BDE, but only by a few kJ/mole.

Moreover, compression of the Co-N_{ax} bond has a modest effect on the structure of the corrin ring, as measured by the corrin fold angle. This provides strong evidence against the mechanochemical trigger mechanism [28,32,35,83,111-113]. Again, Figure 7 quantifies the energetic effect of such constraints. For imidazole and DMB, compression

of the Co-N_{ax} bond leads to an *increase* in the BDE. However, for imidazolate, such compressions could decrease the BDE, but our estimates indicate that the maximum effect is 15 kJ/mole or 20% of the observed rate enhancement by the enzymes.

Similar small effects for neutral ligands have also been observed in earlier theoretical investigations with smaller models or less accurate methods [44,47,52,114-116]. Early semiempirical and extended Hückel calculations gave a stiffer Co-N_{ax} bond [114,115], whereas molecular mechanics simulations indicated that the Co-N_{ax} bond has a slightly smaller effect on the Co-C bond length [47]. In general, theoretical calculations have shown that variations in the Co-N_{ax} bond length in the ground state may contribute at most 1/3 of the enzymatic enhancement of the homolysis rate [44,47,52].

However, it has recently been argued that the main effect of the Co-N_{ax} bond is found only in the transition state and that realistic constraints in the Co-N_{ax} bond length may favour the enzymic reaction for as much as 80 kJ/mole [44]. Yet, this estimate seems to be an artefact of the mixture of molecular mechanics and semiempirical methods (single-point ZINDO energies on MM2-optimised constrained geometries). In fact, the results merely reflect that the optimum ZINDO bond length for the models used is around 1.96 Å, as a calculation at a Co-N_{ax} distance of ~2.23 Å would have shown. This is confirmed by the calculations by Maseras, et al., which show no effect of variations in the Co-N_{ax} bond length along the Co-C reaction coordinate [52].

Thus, we conclude that, irrespectively of the axial base, constraints in the axial Co-N_{ax} bond length can only have a small effect on the strength on the Co-C bond. Instead, we suggest that the proteins enhance the reaction by differential binding [44,109] of the substrate and the Co-C bond-cleavage products, as was discussed above. This can be accomplished by *stabilising* the products (or rather the transition-state structure) by electrostatic and other interactions. In our opinion, it seems much more reasonable and effective if all the difference in binding energy is directly used for stabilisation of the transition state, rather than indirectly by straining the substrate (coenzyme). In a forthcoming paper, we will provide a quantitative analysis of this

suggestion [117].

Acknowledgements

This investigation has been supported by computer resources of the Swedish Council for Planning and Coordination of Research (FRN), Parallelldatorcentrum (PDC) at the Royal Institute of Technology, Stockholm, the High Performance Computing Center North (HPC2N) at the University of Umeå, and Lunarc at Lund University.

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Table 1. Co-ligand distances (in Å) in the optimised complexes.

Corrin	Co-N_{ax}	Co-C	Co-N_{eq}
Co^{III}CorDMBMe$^+$	2.325	1.964	1.890-1.943
Co^{III}CorImMe$^+$	2.250	1.966	1.892-1.946
Co^{III}CorImmMe	2.084	1.991	1.891-1.948
Co^{III}CorDMBRib$^+$	2.388	1.990	1.888-1.944
Co^{III}CorImRib$^+$	2.279	1.992	1.889-1.951
Co^{III}CorImmRib	2.083	2.02	1.891-1.955
Co^{II}CorDMB$^+$	2.318	---	1.896-1.949
Co^{II}CorIm$^+$	2.252	---	1.896-1.948
Co^{II}CorImm	2.127	---	1.885-1.952

Table 2. Bond lengths to cobalt (in Å) in available crystal structures of cobalamin-containing proteins.

Protein	file	Resol.	Co-N_{ax}	Co-C	Co-N_{eq}	Ref.
Methionine synthase	1bmt	3.0	2.14, 2.24	1.96-2.08 (CH_3)	1.91-2.01	4
Methylmalonyl-	1req[a]	2.0	2.49, 2.51	(2.93 to H_2O)	1.56-2.08	5
CoA mutase	2req[a]	2.5	2.67, 2.77	-	1.72-1.94	5
	3req[a]	2.7	2.47	2.03 (Ado)	1.67-1.97	5
	4req[a]	2.2	2.43, 2.47	4.54, 4.57 (Ado)	1.68-1.92	75
	5req[a]	2.2	2.36, 2.43	-	1.67-2.02	118
	6req[a]	2.2	2.46, 2.48	-	1.67-1.97	74
	7req[a]	2.2	2.33, 2.48	(2.60, 2.63 to H_2O)	1.84-1.95	74
	1eic[a]	2.6	2.32, 2.29	(2.74 to H_2O)	1.68-2.00	119
Diol dehydratase	1dio[a]	2.2	2.50, 2.53	-	1.76-2.04	76
	1eex	1.7	2.13, 2.22	1.88, 2.06 (AdePent)	1.85-1.88	120
	1egm	1.85	2.18, 2.27	-	1.84-1.89	120
	1egv	1.75	2.11, 2.23	1.91, 2.16 (AdePent)	1.86-1.88	120
Glutamate mutase	1cb7[b]	2.0	2.34	1.96 (CH_3)	1.88-1.91	6
	1ccw[b]	1.6	2.27, 2.29	1.95 (CN)	1.89-1.93	6
ATP:corrinoid adenosyltransferase	1g64	2.1	2.21	-	1.83-1.90	121

[a] The cobalamin is predominantly in the Co^{II} state.

[b] The cobalamin is a mixture of the Co^{II} and Co^{III} states.

Table 3. Corrin fold angles (in degrees) upon Co-N_{ax} bond displacement.

	Co-N_{ax} displacement (Å)				
Model	-0.30	-0.15	0	0.15	0.30
Co^{III}CorDMBMe^{+}	12.5	10.5	9.0	7.8	6.2
Co^{III}CorImMe^{+}	6.4	5.6	5.3	4.7	4.5
Co^{III}CorImmMe	9.6	8.3	7.1	6.2	5.7
Co^{III}CorDMBRib^{+}	11.2	9.5	8.0	6.6	5.4
Co^{III}CorImRib^{+}	5.5	4.9	4.3	4.1	4.0
Co^{III}CorImmRib	9.2	8.3	7.1	6.0	4.8
Co^{II}CorDMB$^{+\cdot}$	10.5	8.9	7.8	6.6	6.1
Co^{II}CorIm$^{+\cdot}$	6.8	6.1	5.6	5.4	5.3
Co^{II}CorImm$^{\cdot}$	8.9	7.7	6.8	6.2	6.1

Figure 1. The cobalamin system.

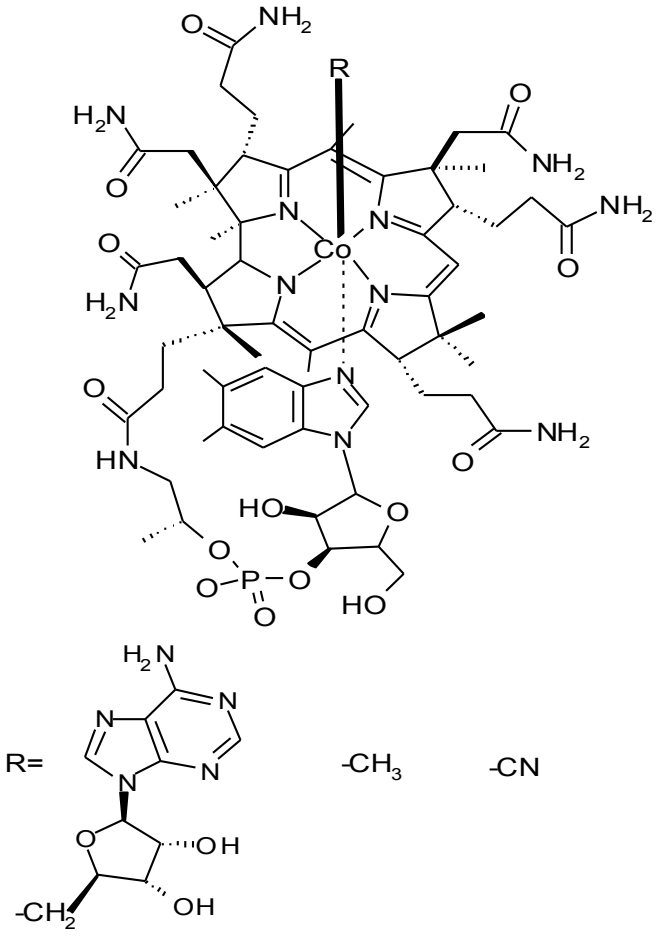
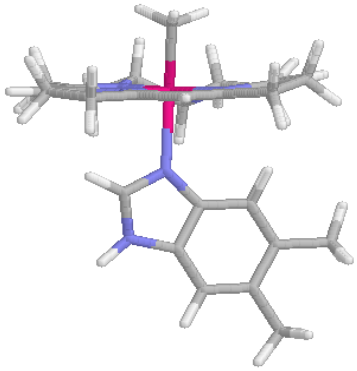
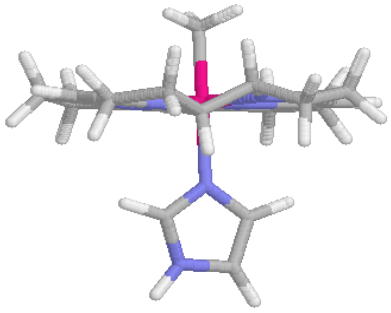


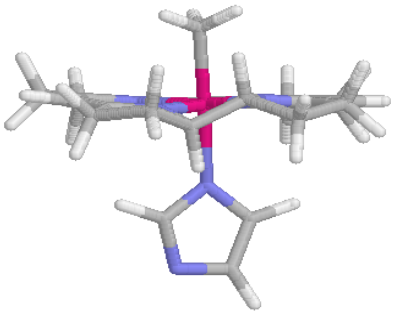
Figure 2. Optimised structures of the investigated corrin models.



a: Co^{III}CorDMBMe^{+}

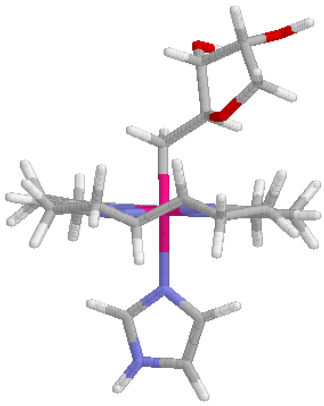


b: Co^{III}CorImMe^{+}

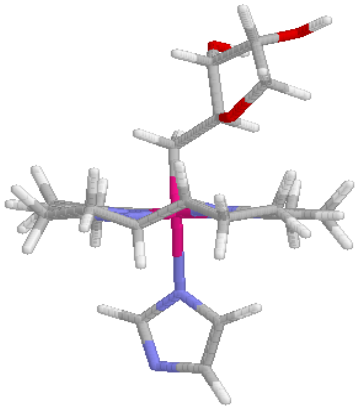


c: Co^{III}CorImmMe

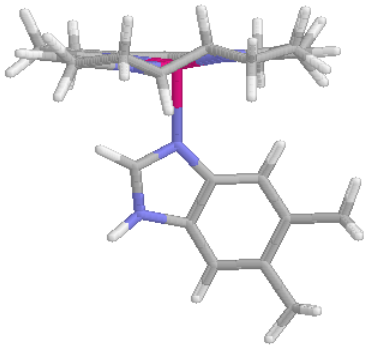
d: Co^{III}CorDMBRib^{+}



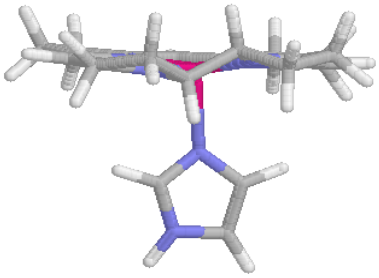
e: $Co^{III}CorImRib^{+}$



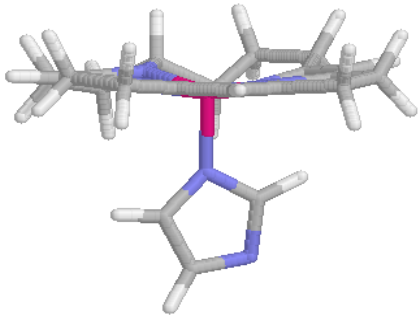
f: $Co^{III}CorImmRib$



g: $Co^{II}CorDMB^{+}$



h: $Co^{II}CorIm^{+}$



i: Co^{II}CorImm

Figure 3. Total energy of the corrin models as a function of Co-N_{ax} bond length. The energy of the equilibrium structure is set to zero for each complex.

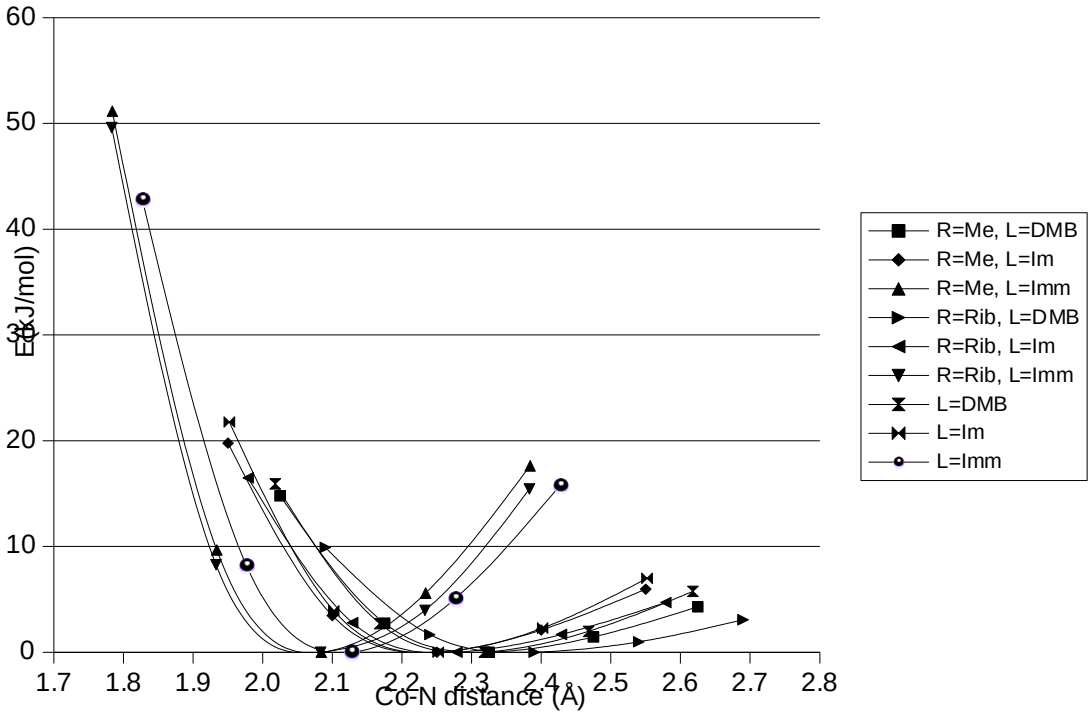


Figure 4. Overlay of the four relaxed structures of $Co^{III}CorImMe^{+}$ (a) and $Co^{III}CorDMBMe^{+}$ (b), with the Co-N_{ax} bond displaced from its optimum value by 0.15 and 0.30 Å.

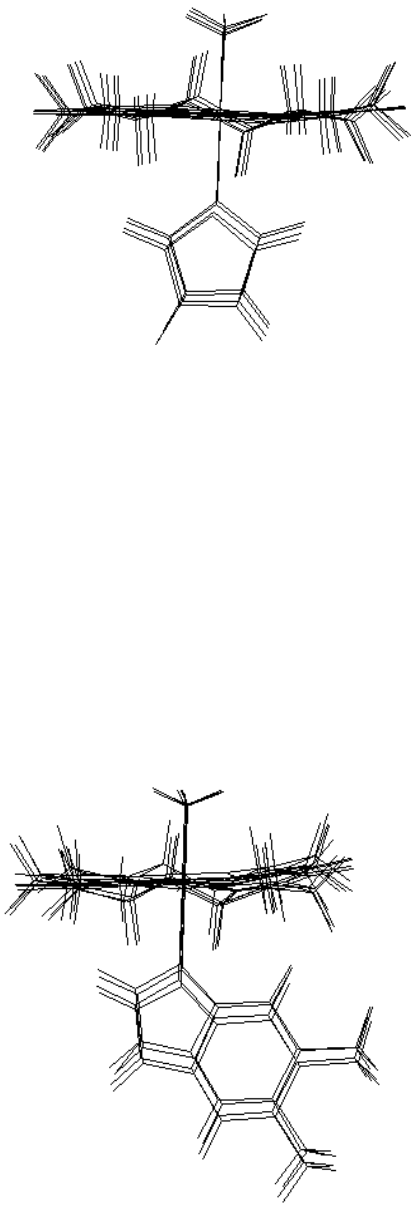


Figure 5. Co-C bond length of octahedral corrins as a function of Co-N_{ax} bond length.

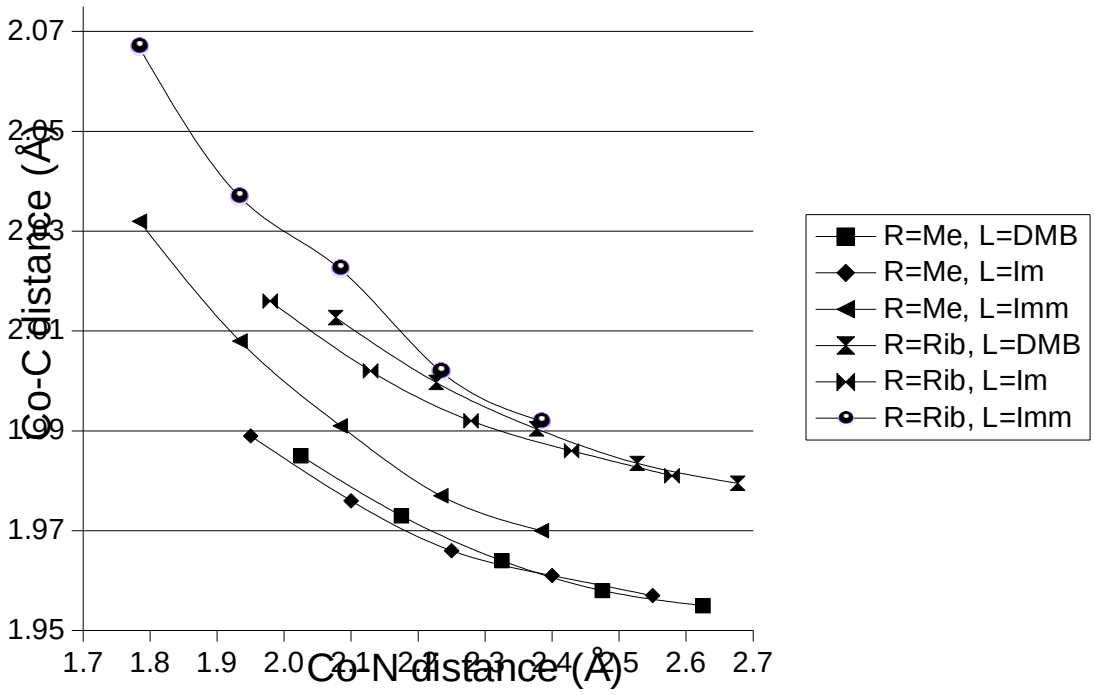


Figure 6. Total energy of $CoCorImMe^{+}$ and $Co^{III}CorImRib^{+}$ as a function of the Co-C bond distance.

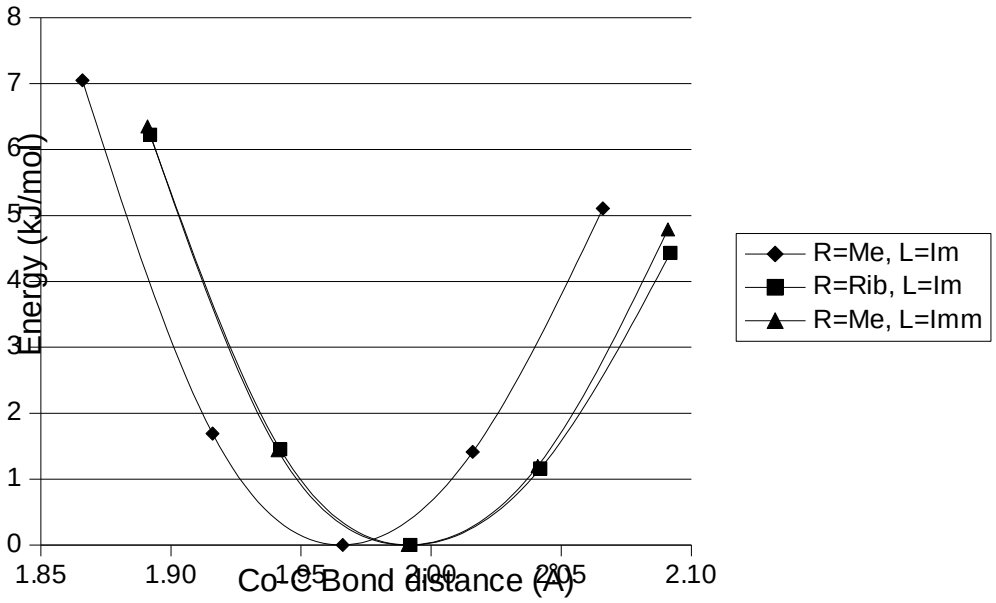


Figure 7. Co-C bond dissociation energy as a function of Co-N_{ax} displacement in the corrins models, calculated with the DZpdf/6-31G* basis set.

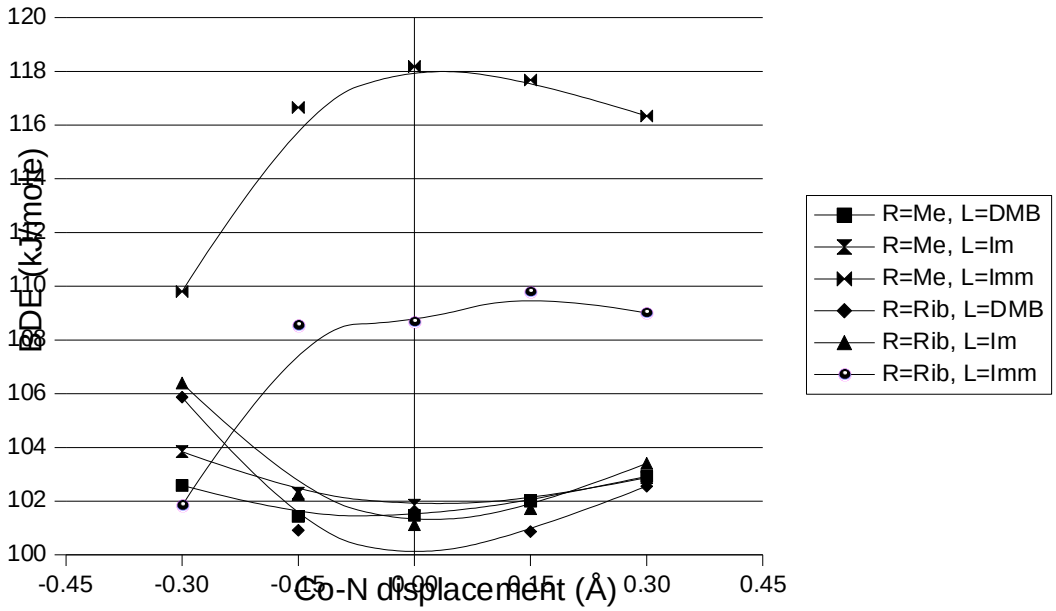


Figure 8. The Co-N_{ax} bond length of $Co^{III}CorImMe^{+}$ and $Co^{III}CorImRib^{+}$ as a function of Co-C bond length.