Assessment of the Existence of Hyper-Long Axial Co(II)−N Bonds in Cobinamide B_{12} Models by Using Electron Paramagnetic Resonance Spectroscopy

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Abstract: Protein control of cobalt-axial nitrogen ligand bond length has been proposed to modulate the reactivity of vitamin B_{12} coenzyme during the catalytic cycle of B_{12}-dependent enzymes. In particular, hyper-long Co−N bonds may favor homolytic cleavage of the *trans*-cobalt−carbon bond in the coenzyme. X-ray crystallographic studies point to hyper-long bonds in two B_{12} holoenzymes; however, mixed redox and ligand states in the crystals thwart clear conclusions. Since EPR theory predicts an increase in Co(II) hyperfine splitting as donation from the axial N-donor ligand decreases, EPR spectroscopy could clarify the X-ray results. However, the theory is apparently undermined by the similar splitting reported for the 2-picoline (2-pic) and pyridine (py) adducts of Co(II) cobinamide (Co(II)Cbi$^+$), adducts thought to have long and normal Co−N axial bond lengths, respectively. Cobinamides, with the B_{12} 5,6-dimethylbenzimidazole loop removed, are excellent B_{12} models. We studied Co(II)Cbi$^+$ adducts of unhindered 4-substituted pyridines (4-X-py's) in ethylene glycol to separate orbital size effects from Co−N axial distance effects on these splittings. The linear increase in splitting with the decrease in 4-X-py basicity found is consistent with the theoretically predicted increase in unpaired electron spin density as axial N lone pair donation to Co(II) decreases. No adduct (and hence no hyper-long Co(II)−N axial bond) was formed even by 8 M 2-pic, if the 2-pic was purified by a novel Co(III)-affinity distillation procedure designed to remove trace nitrogenous ligand impurities present in 2-pic distilled in the regular manner. Adducts formed by impurities in 2-pic and other hindered pyridines misled previous investigators into attributing results to adducts with long Co−N bonds. We find that many 2-substituted py's known to form adducts with simple synthetic Co models do not bind Co(II)Cbi$^+$. Thus, the equatorial corrin ring sterically impedes binding, making Co(II)Cbi$^+$ a highly selective binding agent for unhindered sp^2 N-donor ligands. Our results resolve the apparent conflict between EPR experiment and theory. The reported Co(II) hyperfine splitting of the enzyme-bound cofactor in five B_{12} enzymes is similar to that of the relevant free cofactor. The most reasonable interpretation of this similarity is that the Co−N axial bond of the bound cofactor is not hyper-long in any of the five cases.

Introduction

The relationship of B_{12}-dependent enzymic processes to the nature of cobalt axial ligation in enzyme-bound B_{12}-cofactors has been actively investigated.[1−3] In the holoenzyme, the N-donor axial ligands include dimethylbenzimidazole (DMBz), present in the cobalamin cofactor, and imidazole (Imd), donated by a histidyl residue in certain enzymes. We consider the relationship between the N-donor axial ligand and the catalytic activity of the coenzyme to be the most uncertain aspect of the involvement of the cofactor in the catalytic cycle, especially with respect to Co−C bond homolysis.[1] Physical and chemical properties of the Co center will respond to variations in Co−N bond length. We have noted that the long Co−N axial bond in the cofactors is the only markedly different structural feature compared to synthetic models.[1,4,5] This observation, along with the clear relationship between Co−N bond length and Co−C bond homolysis rate in models,[6] led to our proposal that control of this distance may be crucial in the biological processes.[1] One can imagine that a change in protein conformation can influence the Co−N bond length and hence the reactivity of the cofactor, both in enzymes in which the cofactor retains DMBz ligation[7−9] and in enzymes in which the cofactor shows protein histidyl ligation.[10,11] It has been proposed that Nature's goal in utilizing a weakly bound axial ligand such as DMBz may be to limit

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Co−C bond heterolysis and promote the more biologically relevant homolysis in AdoCbl-dependent enzymatic reactions.[12,13]

These proposals have gained significance from the recent finding that the Co−N(Imd) bond in methylmalonyl-CoA mutase may be hyper-long (reported as 2.53 Å).[11] More recently, the Co−N(DMBz) bond in diol dehydrase was also reported to be long (2.50 Å).[14] However, the size of the enzymes, the mixed Co redox and ligand states, and the instability of the crystals make assessment of this distance by X-ray methods difficult.[15] EXAFS methods[15−17] have also been employed in defining the Co−N axial bond length in the cofactors, but the accuracy of this method is limited by the presence of four equatorially bound corrin nitrogens, which can interfere with this measurement.[16,18] Since it would be useful to have a means of calibrating the relationship of spectral features to the Co−N axial bond length, we are exploring the influence of the axial ligand on spectroscopic properties that can be measured in holoenzymes. There are now many examples of the use of spectroscopy for correlating the properties of the B_{12} models, including cobalamins, to parameters such as axial Co−N bond length.[19−21] In order for the spectroscopic changes in models to be directly applied to the interpretation of the related data for cofactors in enzymes, the models must have structural features very similar to those of the bound cofactor. The best-studied model systems closely related to cobalamins are cobinamides (Cbi^+'s, Figure 1). Cbi^+'s are derivatives of the cobalamin cofactors which have the axial benzimidazole nucleotide chemically removed, making them useful models for examining the binding characteristics of exogenous nitrogenous ligands. Consequently, the Co−N bond length might be systematically varied by varying N-donor ligand bulk, and the nature of the electron pair donation can be varied by changing ligand basicity. Such studies have led to many observations relevant to the role of the axial N-donor ligand in B_{12}-dependent enzymes.[22] However, a major impediment to Cbi^+ studies is the generally weak axial ligand binding to both Co(II)Cbi^+ and RCo(III)Cbi^+ (Figure 1) and the relative paucity of information to guide our study based on relevant Co(II) synthetic models. In the present study, we selected electronic absorption and EPR spectroscopies, two methods applicable to enzyme systems, to examine axial ligation to Co(II)Cbi^+'s. In the short term, we needed to determine the factors that govern the ability or inability of nitrogenous ligands to form detectable adducts with Co(II)Cbi^+. In the long term, the results will provide benchmarks against which to compare the cofactors in the active site of enzymes.

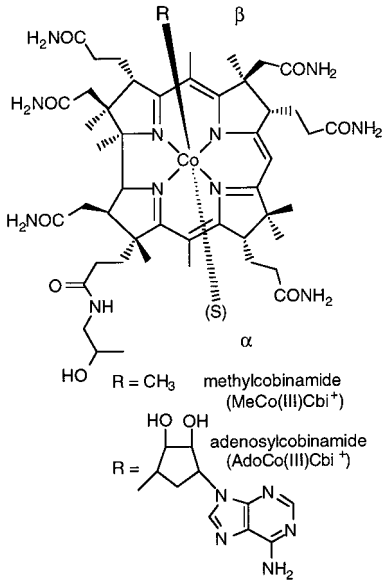
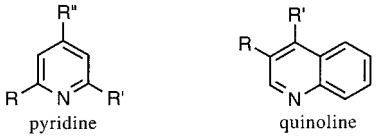


Figure 1. Structure of $RCbi^+$. The presence of axial solvent (S) is uncertain. Co(II)Cbi^+ used in this study has the axial alkyl (R) group removed upon photolysis in the absence of ambient oxygen. In the Co(II)Cbi^+ form, exogenous N-donor ligands may bind at the upper (β) or lower (α) side of the corrin.

Table 1. Structures and Abbreviations of the N-Donor Ligands Used in This Study



N-donor ligand	abbreviation	R	R′	R″
Unhindered Ligands				
pyridine	py	H	H	H
4-cyanopyridine	4-CNpy	H	H	CN
4-picoline (4-methylpyridine)	4-pic	H	H	CH_3
4-(dimethylamino)pyridine	4-Me_2Npy	H	H	$N(CH_3)_2$
Hindered Ligands				
quinoline	quin	H	H	−
3-methylquinoline	3-Me-quin	CH_3	H	−
4-methylquinoline	4-Me-quin	H	CH_3	−
2-vinylpyridine	2-vinylpy	$CHCH_2$	H	H
2-picoline (2-methylpyridine)	2-pic	CH_3	H	H
2,6-lutidine (2,6-dimethylpyridine)	2,6-lut	CH_3	CH_3	H
2-aminopyridine	2-NH_{2py}	NH_2	H	H
2-methoxypyridine	2-MeOpy	OCH_3	H	H
2-methylaminopyridine	2-MeNHpy	$HNCH_3$	H	H

We selected pyridine N-donor ligands since these could be systematically varied in both electronic and steric properties (see Table 1 for ligand structures and abbreviations). The unhindered, similarly sized 4-substituted pyridine derivatives were studied to assess what effect variation in electron-donating ability of the ligand has on the Co(II) hyperfine and ligand ^{14}N superhyperfine splitting measured in the EPR spectrum. This allows resolution of "orbital size" effects from Co−N distance effects on these splittings. The sterically bulky pyridine-type ligands, having at least one substituent *ortho* to the pyridine nitrogen, were selected to assess the effect of increasing Co−N bond length on the splittings. Two main factors suggested the utility of these sterically hindered pyridine-type ligands: First, both visible[22] and EPR[23,24] data have been interpreted to suggest that bulky pyridines bind to Co(II)Cbi^+. Second, calculations[22] as

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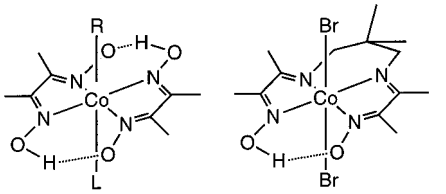
Chart 1. Structures of $LCo(DH)_2R$ and $Co((DO)(DOH)Me_2Pn)Br_2$ Model Compounds



$LCo(DH)_2R$ $Co((DO)(DOH)Me_2pn)Br_2$

well as a simple inspection of molecular models indicated that such ligands could not form Co−N axial bonds of normal length. The work proposing these long bonds[22] cited the fact that bulky pyridines are known to give rise to a long Co−N bond in cobaloxime models (Chart 1, $LCo(DH)_2R$, where DH = monoanion of dimethylglyoxime, R = mononegative alkyl or aryl, and L = neutral ligand).[25] The study of $AdoCbi^+$ (Figure 1) models with bulky ligands has led to the suggestion that the influence of the axial ligand occurs mainly in a Co(II)-like transition state.[22]

In ethylene glycol, the solvent used in this and most studies,[22,23,26−31] Co(II) in the Cbi^+ species is accepted as being five-coordinate with one axial solvent ligand.[23] This form is referred to as $Co(II)Cbi^+$. The five-coordinate species with an N-donor axial ligand is designated as $LCo(II)Cbi^+$. Early in our study, we observed that addition of hindered pyridines caused changes in the EPR spectrum of $Co(II)Cbi^+$. These changes were similar to those in the literature, but we suspected that the results were due to trace impurities in the ligands. We have developed a generally applicable novel distillation technique utilizing a Co(III) "affinity reagent" that selectively traps trace unhindered nitrogenous ligand impurities present in sterically hindered ligands.

Experimental Section

Materials. Anhydrous ethylene glycol and N-donor ligands (see Table 1 for ligand abbreviations) were obtained from Aldrich. Other samples of quin and 2-vinylpy were subsequently obtained from Lancaster. Methylaquacobinamide$^+$ ($MeCbi^+$) was prepared and isolated as the Cl^-, ClO_4^-, and PF_6^- salts as previously reported.[32,33] *Caution! Perchlorate salts may be explosive and should be handled in small quantities with appropriate precautions.* The visible spectrum of $MeCbi^+$ in ethylene glycol has a maximum at 461 nm, in agreement with literature values.[34,35] The ^1H NMR spectrum of $MeCbi^+$ in D_2O shows only one downfield signal (6.76 ppm, referencing to HOD at 4.80 ppm) for the proton attached directly to the corrin ring at C10. This result verified complete removal of the benzimidazole nucleotide since the downfield signals for the protons on the benzimidazole and ribose rings were no longer observable.[36] The imine/oxime complex, $Co((DO)(DOH)Me_2pn)Br_2$ (Chart 1), was prepared as previously reported.[37]

Distillation Procedure. 2-Pic (Table 1) was distilled after addition of ~0.2 M $Co((DO)(DOH)Me_2pn)Br_2$ using a bulb-to-bulb technique under vacuum and at temperatures <40 °C. Three consecutive distillation cycles were performed because of solubility limitations of $Co((DO)(DOH)Me_2pn)Br_2$; the ligand, collected in a round-bottomed flask placed in a dry ice/acetone bath, was stored in a sealed flask under refrigeration.

Photolysis of $MeCbi^+$ to $Co(II)Cbi^+$. For EPR spectroscopy, solutions of $MeCbi^+$ in ethylene glycol in 4 mm OD EPR tubes (1 mM, 300 μL) containing either no N-donor ligand or 0.005−9.2 M ligand were deaerated in the dark with 3−4 successive freeze−pump−argon gas cycles. Samples were irradiated with the water- and glass-filtered output of a 1 kW Hg−Xe arc lamp (Oriel) placed ~12 in. away. The solution was immediately frozen to a glass by immersing the EPR tube in liquid nitrogen. Photolysis of 1 and 10 mM $MeCbi^+$ solutions was found to proceed to >95% completion within 30 s and 7.5 min of irradiation, respectively, determined by monitoring the intensity of the EPR signal of $Co(II)Cbi^+$ with time. Samples were irradiated 90 s and 10 min, respectively, to ensure complete photolysis.

For visible spectroscopy, samples of $Co(II)Cbi^+$ were prepared by irradiation of 100 μM solutions of $MeCbi^+$ in ethylene glycol. Prior to photolysis, the $MeCbi^+$ solutions in a 1 cm Schlenk cuvette were purged with N_2 in a Vacuum Atmospheres glovebox. Photolysis was performed by exposing the sample to a 75 W light placed ~8 in. from the sample to minimize heating and was complete within 40−45 min of irradiation, evident by the absence of further spectral change. The final absorbance maximum of $Co(II)Cbi^+$ was 468−469 nm, and the intensity of this band was consistent with the reported molar absorptivity value for $Co(II)Cbi^+$.[38]

EPR Spectroscopy. EPR spectra were obtained using a Bruker 4102ST TE102 cavity, HP 4256L frequency counter, Varian V3603 electromagnet and Fieldial Mark I regulator/power supply, and an Air Products cryostat and temperature controller modified for nitrogen gas flow sample cooling at 120 K. Spectra were acquired at 9.45 GHz with a modulation depth of 10 G and microwave power of 20 mW, and 2−4 spectra were averaged. For measurement of hyperfine and superhyperfine splittings, 10 scans of the third through the fifth hyperfine features of the octet were averaged over a 350 G range from 3150 to 3500 G. A baseline EPR spectrum of an ethylene glycol glass at 120 K was subtracted from each spectrum obtained at 120 K for glasses containing $Co(II)Cbi^+$ or $LCo(II)Cbi^+$.

Visible Spectroscopy. Titrations of $Co(II)Cbi^+$ (100 μM in ethylene glycol) with neat N-donor ligands (up to 2.0 M) were monitored by visible spectroscopy using a Varian Cary 3 spectrophotometer. In the glovebox, all ligands were purged with N_2 and then added to the $Co(II)Cbi^+$ solution. Spectra were corrected for dilution by using the linear relationship between percent ligand added and the total volume, determined experimentally.

Results

EPR Spectroscopy of $Co(II)Cbi^+$ in the Absence of N-Donor Ligand. The EPR spectrum of $Co(II)Cbi^+$ in ethylene glycol glass at 120 K has a typical eight-line hyperfine splitting pattern centered about the $g_{||}$ position at $g = 2.0$ (Figure 2b), which arises from the interaction between the unpaired electron on Co(II) and the $I = {}^7/_2$ cobalt nucleus. The magnitude of the Co(II) hyperfine splitting is 14.20 mT. The spectrum agrees well with literature reports.[23]

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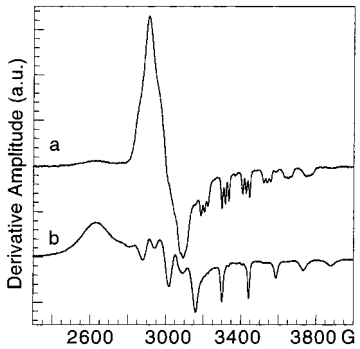

Figure 2. EPR spectra of 1 mM Co(II)Cbi$^+$ in ethylene glycol containing (a) 10 mM py and (b) alone (i.e., in the absence of added N-donor ligand); a.u. = arbitrary units.

Table 2. EPR Hyperfine and Superhyperfine Splittings (±0.017 mT) Obtained for LCo(II)Cbi$^+$ Adducts (1 mM) in Ethylene Glycol[a]

N-donor ligand (L)[b]	pK_a[c]	hyperfine splitting (mT)	superhyperfine splitting (mT)
4-CNpy	1.9	11.32	1.83
py	5.2	11.04	1.83
4-pic	6.0	11.01	1.88
4-Me_2Npy	9.7	10.68	1.98

[a] The hyperfine splitting for Co(II)Cbi$^+$ alone is 14.20 mT. [b] 0.1 M L; definitions of abbreviations are given in Table 1. [c] pK_a values obtained from ref 39.

EPR Spectroscopy of Co(II)Cbi$^+$ Adducts of Unhindered 4-X-Pyridines (X = CN, H, CH_3, NMe_2). Binding to Co(II)Cbi$^+$ of unhindered 4-substituted pyridine derivatives (4-X-py, Table 1) with a wide range of basicity was studied by EPR spectroscopy to assess (a) the concentrations needed to bind Co(II)Cbi$^+$ completely and (b) the effect of a variation in electron-donating ability of L on the hyperfine and superhyperfine splitting of LCo(II)Cbi$^+$. An EPR spectrum of 1 mM Co(II)Cbi$^+$ in ethylene glycol containing 5 mM py has mainly the signal for pyCo(II)Cbi$^+$ with a small signal for unbound Co(II)Cbi$^+$. When the concentration of py was increased to 10 mM, the signal from pyCo(II)Cbi$^+$ increased, while that of unbound Co(II)Cbi$^+$ decreased even more to become barely visible above the baseline noise (Figure 2a). Binding of only one pyridine ligand per Co(II)Cbi$^+$ was confirmed by the splitting of each hyperfine feature into a superhyperfine triplet due to coupling between the unpaired electron on Co(II) and a single $I = 1$ ^{14}N nucleus. The hyperfine splitting for pyCo(II)Cbi$^+$ was 11.04 mT (Table 2). A more complicated pseudoquintet coupling has been reported to indicate binding of two axial pyridine ligands to Co(II)Cbi$^+$ in water when the amount of pyridine was >50 vol %.[24] However, only superhyperfine triplets were found in spectra obtained here, even in neat pyridine.

In the presence of 0.1 M 4-Me_2Npy or 4-CNpy (Table 1), respectively strong (pK_a = 9.7) and weak (pK_a = 1.9) ligands, complete formation of LCo(II)Cbi$^+$ was observed, and the respective hyperfine splittings were 10.68 mT and 11.32 mT (Table 2). These hyperfine values bracket those measured for pyCo(II)Cbi$^+$ and 4-picCo(II)Cbi$^+$ (Table 2). Plotting the 4-X-pyCo(II)Cbi$^+$ hyperfine splittings against the pK_a values of the 4-X-py ligands gave a straight line with a correlation factor of $R = 0.998$ (Figure 3). This finding shows that the 4-X-pyCo(II)Cbi$^+$ hyperfine splitting increased as the donor ability of 4-X-py decreased in the order 4-Me_2Npy > 4-pic > py > 4-CNpy (Table 2). No large difference in superhyperfine splittings was observed among these complexes, except for 4-Me_2NpyCo(II)Cbi$^+$, which gave a larger superhyperfine value (1.98 mT) than those of the other LCo(II)Cbi$^+$ adducts (Table 2). The superhyperfine splitting for 4-Me_2NpyCo(II)Cbi$^+$ was distinct even at the seventh and eighth hyperfine features of the octet at high field (~3600−3800 G), whereas the other LCo(II)Cbi$^+$ complexes showed broader superhyperfine features in this region of the EPR spectrum. No quintet splitting was observed in 3 M 4-Me_2Npy, indicating that with a high concentration of an even better ligand than pyridine, only one 4-Me_2Npy ligand binds to Co(II)Cbi$^+$.

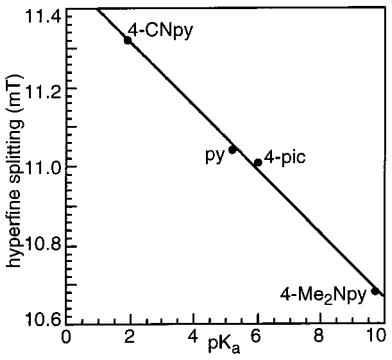

Figure 3. Plot of EPR hyperfine splitting as a function of pK_a of 4-X-py for 4-X-pyCo(II)Cbi$^+$.

EPR Spectroscopy of Co(II)Cbi$^+$ in the Presence of Hindered Ligands. Quinoline. EPR experiments showed that in the presence of 2.2 M quin (first batch tested) Co(II)Cbi$^+$ formed a considerable amount of an LCo(II)Cbi$^+$ species (with signals remaining for Co(II)Cbi$^+$). However, 3-Me-quin and 4-Me-quin, quinoline derivatives (Table 1) having an electron-donating but not sterically hindering methyl substituent, formed no LCo(II)Cbi$^+$ species even at 3 M. The 3-Me-quin and 4-Me-quin experiments gave EPR spectra (not shown) essentially identical to that of solvated Co(II)Cbi$^+$ (Figure 2b). These results suggested strongly that impurities in quin were actually binding. However, the high-temperature-distilled quin gave only very small ^{1}H NMR signals that could be from an impurity. EPR spectra of solutions with 1 M high-temperature-distilled quin were recorded at *10 mM* and *1 mM* Co(II)Cbi$^+$ concentrations. The relative amplitudes of the superhyperfine features versus the hyperfine features of the unbound Co(II)Cbi$^+$ at 10 mM vs 1 mM Co(II) would remain the same if the superhyperfine features were due to a quinCo(II)Cbi$^+$ species since the quin is present in great excess. The relative amplitude would decrease if the superhyperfine features were from an (impurity)Co(II)Cbi$^+$ species formed by <10 mM impurity. The relative amplitude of these features in the 10 mM Co(II)Cbi$^+$ sample was only slightly smaller than that of the features in the 1 mM Co(II)Cbi$^+$ sample (not shown). However, the decrease was slight, and this experiment was not decisive. Therefore, a sample was prepared with 1 M quin obtained from a different supplier (and distilled). In contrast to the results obtained with the first batch of quin, no Co(II)Cbi$^+$ signals remained. Only (impurity)-Co(II)Cbi$^+$ EPR signals were observed. The hyperfine splitting was 11.17 mT, the same value as for the first batch. Unlike for the first batch, the second batch of quin (after distillation) gave impurity ^{1}H NMR signals that were large enough to integrate. These signals indicated the presence of 2−3% impurity. Thus, in the sample of the second batch of quin (1 M), 20−30 mM impurity was present.

Since nitrogenous impurities in quin, not quin itself, bind to Co(II)Cbi$^+$ and since py and quin have similar basicity (5.2 vs 4.8, respectively),[39] it is clear that steric effects greatly impede axial ligand binding to Co(II)Cbi$^+$. These experiments with quin also indicate that, in cases requiring a large excess of ligand to

produce LCo(II)Cbi$^+$ adducts, distinguishing between ligand and impurity binding requires special care.

2-Vinylpyridine. EPR experiments with 2-vinylpy similar to those with quin showed the presence of signals for both an LCo(II)Cbi$^+$ species and Co(II)Cbi$^+$. Because of the possibility that the LCo(II)Cbi$^+$ species was formed by nitrogenous impurities in commercial 2-vinylpy, we tested two other batches of 2-vinylpy (one from a different supplier) and found that the intensity of the superhyperfine features for LCo(II)Cbi$^+$ differed considerably in 2.3 M 2-vinylpy samples. In addition, the observed hyperfine splitting measured for the LCo(II)Cbi$^+$ species varied considerably with the 2-vinylpy batch (11.07 to 11.15 mT). Thus, commercial supplies of 2-vinylpy contain impurities in varying amounts and identity. To assess this conclusion further, we performed a similar set of experiments with the concentration of Co(II)Cbi$^+$ varied over a large range. At 3 M 2-vinylpy and 0.2, 1, and 15 mM Co(II)Cbi$^+$, the relative amplitude of the superhyperfine features for LCo(II)Cbi$^+$ compared to the hyperfine features for unbound Co(II)Cbi$^+$ decreased, further demonstrating that impurities, not the 2-vinylpy ligand itself, formed the LCo(II)Cbi$^+$ species.

2-X-pyridines. In a survey of other potential (2-X-py)Co(II)Cbi$^+$ adducts, we prepared Co(II)Cbi$^+$ ethylene glycol solutions with 1 M 2-pic, 2-NH_2py, 2-MeOpy, and 2-MeNHpy (Table 1). EPR spectra of glasses formed from these solutions contained dominant signals of Co(II)Cbi$^+$ with at best small superhyperfine features for LCo(II)Cbi$^+$ species. Thus, none of these 2-X-py's could be a good ligand, and studies at high enough concentration of 2-X-py to obtain a measure of the hyperfine splitting will raise the possibility that better-binding nitrogenous impurities in these sterically hindered ligands actually form the adducts responsible for the small superhyperfine features. It would be impractical to conduct exhaustive studies of each 2-X-py ligand. Therefore, we focused our attention on the 2-pic ligand for several reasons. First, two earlier studies have reported that it binds to Co(II)Cbi$^+$ (observed by visible spectroscopy in ethylene glycol[22] and by EPR spectroscopy in water[24]). Also, one other report refers to unpublished work in which 2-picCo(II)Cbi$^+$ was detected by EPR spectroscopy.[23] Second, the 2-pic and 2,6-lut (Table 1) ligands play a key role in recent proposals on the influence of the axial N-donor ligand on Co−C bond homolysis (see below).[22] Third, crystallographic results for synthetic B_{12} models show a similarly long Co−N axial bond for the 2-pic and the 2-vinylpy adducts (unpublished studies). Fourth, from EPR results (next paragraph), 2-pic appeared to have a low level of impurity.

2-Picoline. In the presence of 1 M 2-pic distilled once at high temperature, we observed no superhyperfine splitting in the EPR spectrum to indicate a nitrogen ligand bound to Co(II)Cbi$^+$ (Figure 4b). This spectrum is almost identical to that obtained in the presence of 1 M 2,6-lut (Figure 4c), an even more sterically demanding ligand. Both spectra are essentially identical to that of solvated Co(II)Cbi$^+$ (Figure 2b). The EPR spectrum of Co(II)Cbi$^+$ in the presence of 1 M py (showing 100% pyCo(II)Cbi$^+$) is reproduced in Figure 4a for comparison.

In addition to 1 M 2-pic (Figure 4b), EPR spectra of Co(II)Cbi$^+$ in ethylene glycol were acquired in the presence of 5, 7, 8, and 9.2 M 2-pic. Not until the concentration of 2-pic was at least 8 M were superhyperfine signals of considerable intensity observed, indicating the presence of one or more LCo(II)Cbi$^+$ complexes containing an axially bound N-donor (Figures 5a and 6a). At 9.2 M 2-pic (almost neat 2-pic with only 9% (v/v) ethylene glycol needed to solubilize the MeCbi$^+$ prior to photolysis), the signals for LCo(II)Cbi$^+$ increased such that the corresponding superhyperfine features became slightly larger in amplitude than the separate hyperfine signals for unbound Co(II)Cbi$^+$ (Supporting Information). To assess for the possible presence of impurities, the EPR spectra of *10 mM* and *1 mM* Co(II)Cbi$^+$ solutions with 8 M high-temperature-distilled 2-pic were recorded. The superhyperfine features for LCo(II)Cbi$^+$ are relatively much smaller in the 10 mM than in the 1 mM Co-

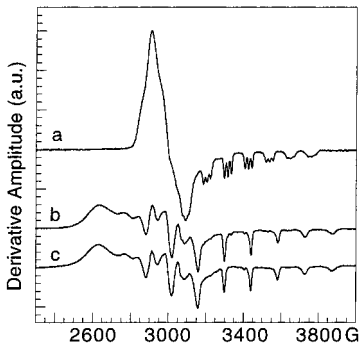

Figure 4. EPR spectra of 1 mM Co(II)Cbi$^+$ in ethylene glycol containing 1 M (a) py, (b) 2-pic, and (c) 2,6-lut. Spectra (b) and (c) are essentially identical to that of Co(II)Cbi$^+$ alone in ethylene glycol (Figure 2b); a.u. = arbitrary units.

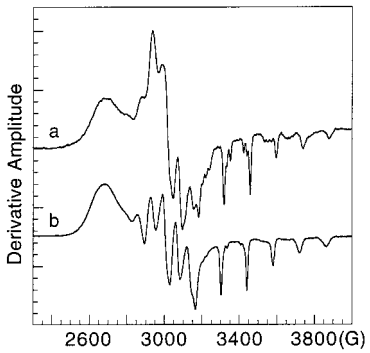

Figure 5. EPR spectra of 1 mM Co(II)Cbi$^+$ in 8 M 2-pic/ethylene glycol: (a) 2-pic distilled once at high temperature and (b) 2-pic after three cycles of Co(III)-affinity distillation.

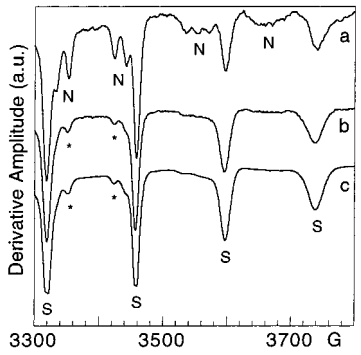

Figure 6. EPR spectra in 8 M 2-pic/ethylene glycol (including expanded versions of spectra in Figure 5): (a) 1 mM Co(II)Cbi$^+$, 2-pic distilled once at high temperature; (b) 1 mM Co(II)Cbi$^+$, 2-pic after three cycles of Co(III)-affinity distillation; and (c) 10 mM Co(II)Cbi$^+$, 2-pic distilled once at high temperature. The *y* axes of spectra (a) and (b) have been increased 10-fold to allow comparison to spectrum (c); a.u. = arbitrary units. N = Co(II)Cbi$^+$ with N-donor bound and S = solvated Co(II)Cbi$^+$ with no N-donor bound. The asterisks near $g = 2$ mark very small features found even in samples of Co(II)Cbi$^+$ in the absence of added N-donor ligand (Figure 2b).

(39) Christensen, J. J.; Hansen, L. D.; Izatt, R. M. *Handbook of Proton Ionization Heats and Related Thermodynamic Quantities*; John Wiley & Sons: New York, 1976.

(II)Cbi$^+$ sample (Figure 6); indeed, these features are now hardly visible above the noise level in the spectrum of the 10 mM Co(II)Cbi$^+$ sample (Figure 6c). In contrast, the hyperfine signals for unbound Co(II)Cbi$^+$ are of considerably larger amplitude in spectra of the 10 mM versus the 1 mM Co(II)Cbi$^+$ sample. This finding indicated that the ligand which binds to Co(II)-Cbi$^+$ is indeed either one or a mixture of impurities present as a limited percentage of 2-pic.

Co(III)-Affinity Distillation. Since distillation of commercial 2-pic does not appear to remove trace impurities[40,41] and since this ligand was of such pivotal importance in previous studies in the literature,[22−24] we developed a technique to remove Co-binding impurities from commercial 2-pic. The method involved addition of a Co(III) complex to the 2-pic to coordinate any such impurities prior to low-temperature bulb-to-bulb distillation. We chose the imine/oxime complex, Co((DO)(DOH)Me_2pn)-Br_2 (Chart 1), because of its solubility in 2-pic and the proven ability of this type of complex to form robust complexes with unhindered nitrogenous ligands.[42]

When dissolved in 2-pic, the originally green Co((DO)(DOH)-Me_2pn)Br_2 turns red, indicating that some binding has occurred. When 2-pic saturated in Co((DO)(DOH)Me_2pn)Br_2 (∼0.2 M) was distilled (bulb-to-bulb) once under vacuum without high heat, the 2-pic collected no longer gave a red-colored solution with Co((DO)(DOH)Me_2pn)Br_2, indicating that most of the Co-binding impurities had been removed. When the Co(III)-affinity-distilled 2-pic was then used in an EPR experiment at both 8 and 9.2 M concentrations in ethylene glycol, signals for LCo-(II)Cbi$^+$ had decreased in comparison to the original spectrum obtained when using 2-pic distilled in the usual high-temperature manner. After each successive Co(III)-affinity distillation of 2-pic, smaller EPR signals for LCo(II)Cbi$^+$ were observed in the EPR spectrum at either 8 or 9.2 M 2-pic. Following three cycles of Co(III)-affinity distillation, LCo(II)Cbi$^+$ EPR signals were no longer observed even at 8 M (Figures 5b and 6b) or 9.2 M 2-pic (not shown). In addition, the overall shape and intensity of the Co(II)Cbi$^+$ signal was essentially identical to that of solvated Co(II)Cbi$^+$ in ethylene glycol in the absence of added N-donor ligand (Figure 2b), and the hyperfine splitting was 13.90 mT. This value is close to that obtained for solvated Co(II)Cbi$^+$ in ethylene glycol (14.20 mT), the slight difference being attributed to solvent effects.[23]

The observation that successive Co(III)-affinity distillations of 2-pic decreased the amplitude of the superhyperfine features due to LCo(II)Cbi$^+$ indicated one of two possibilities: either (i) the Co((DO)(DOH)Me_2pn)Br_2 removed small amounts of trace nitrogenous species which bind to Co(II)Cbi$^+$, or less likely, (ii) the 2-pic itself was degraded during the distillation, either converting it to a nonbinding species or generating a species which prevents Co(II)Cbi$^+$ from binding to the undegraded 2-pic. Experiments were performed to test the unlikely occurrence of degradation. First, examination of the ^{1}H NMR spectrum of the Co(III)-affinity-distilled 2-pic showed that it was free of water and unaltered. Second, a crystal structure of a cobaloxime complex formed using the affinity-distilled 2-pic reveals an intact bound 2-pic (unpublished studies). Third, an EPR spectrum of 1 mM Co(II)Cbi$^+$ containing 5 mM py in the presence of 8 M Co(III)-affinity-distilled 2-pic showed signals for 100% pyCo(II)Cbi$^+$, having exactly the same hyperfine splitting as observed in ethylene glycol alone, 11.04 mT (Table 2). An EPR spectrum of 1 mM Co(II)Cbi$^+$ in the presence of 5 mM py alone showed signals for almost 100% pyCo(II)Cbi$^+$, but very small signals for unbound Co(II)Cbi$^+$ were still present. These results showed that there was nothing in the sample of Co(III)-affinity-distilled 2-pic preventing axial ligation of Co-(II)Cbi$^+$ by py. The fact that more pyCo(II)Cbi$^+$ was observed in the presence of 8 M 2-pic compared to the sample in ethylene glycol alone is undoubtedly due to the lower amount of ethylene glycol present to compete with py for the axial site.

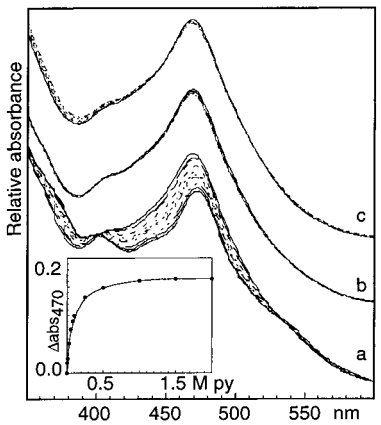

Figure 7. Visible spectra obtained in a titration of 100 μM Co(II)-Cbi$^+$ with up to 2.0 M py (a), 2-pic (b), and 2,6-lut (c) in ethylene glycol. Inset shows change in absorbance (Δ_{abs}) at $\lambda = 470$ nm as a function of py concentration from spectra in (a).

Visible Spectroscopy. Binding of Pyridines to Co(II)Cbi$^+$. Titration of 100 μM Co(II)Cbi$^+$ with py (0.005−2.0 M) in ethylene glycol resulted in a decrease in absorption and shift of the maximum from 469 to 472 nm, along with an increase in absorption at ∼545 nm (Figure 7a). The spectral changes ceased at ∼0.50 M py (Figure 7a, inset), indicating complete formation of pyCo(II)Cbi$^+$. Isosbestic points were present at 342, 399, and 532 nm throughout the titration (Supporting Information). These spectral changes demonstrate the binding of only one pyridine in an axial position. The resulting spectrum is consistent with this finding since it is almost identical to the known spectrum of cob(II)alamin[24] (which contains an axial DMBz ligand). Of note, the absence of even a hint of both a strong γ band at 362 nm and a more intense α band between 500 and 550 nm, which appear in the presence of dioxygen and an unhindered N-donor ligand (see below),[35] confirm that the spectra arise from Co(II) species.

In contrast to the spectral changes that occur upon binding of py to Co(II)Cbi$^+$, no significant changes were found upon addition of either 2-pic (Figure 7b) or 2,6-lut (Figure 7c) to Co(II)Cbi$^+$ over a 0.05−2.0 M concentration range. These visible spectroscopic results obtained at room temperature and ≤2 M ligand support our EPR spectroscopic results obtained at low temperature (120 K) and high concentration of ligand (8 M Co(III)-affinity-distilled 2-pic, Figure 5b). Both types of spectra clearly establish that highly hindered pyridine derivatives such as 2-pic and 2,6-lut are unable to bind to Co(II)Cbi$^+$.

Binding of Pyridines to (Disolvato)Co(III)Cbi^{2+}. We noticed that the visible spectrum of ethylene glycol solutions containing Co(II)Cbi$^+$ and pyridine ligands changed on exposure to air. The resulting spectrum had spectral features similar to features reported[22] for some putative Co(II)Cbi$^+$ adducts of pyridine ligands. We examined the effects of dioxygen on ethylene glycol solutions. Cobinamide solutions were prepared either by photolysis of MeCbi$^+$ in the presence of ambient dioxygen (Figure 8) or by ambient dioxygen exposure to a Co-

(40) Brown, H. C.; Johnson, S.; Podall, H. *J. Am. Chem. Soc.* **1954**, *76*, 5556−5557.
(41) Walker, F. A. *J. Magn. Reson.* **1974**, *15*, 201−218.
(42) Parker, W. O.; Bresciani-Pahor, N.; Zangrando, E.; Randaccio, L.; Marzilli, L. G. *Inorg. Chem.* **1985**, *24*, 3908−3913.

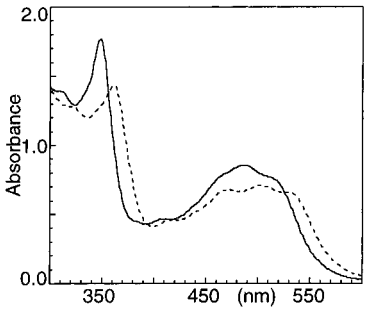

Figure 8. Visible spectra obtained after photolysis of 100 μM MeCbi$^+$ in ethylene glycol while exposed to air to form (disolvato)Co(III)Cbi^{2+} (—), followed by addition of 10 mM py (- - -).

(II)Cbi$^+$ solution. The visible spectrum of either type of solution closely resembles that of (diaqua)Co(III)Cbi^{2+} in water.[43] When an excess of CN^- was added to a solution of the oxidized cobinamide in ethylene glycol, the resulting visible spectrum closely resembled that of a commercial sample of $(CN)_2$Co(III)Cbi in ethylene glycol (Supporting Information). Thus, it is clear that the major product resulting from dioxygen exposure of Co(II)Cbi$^+$ in ethylene glycol is the (disolvato)Co(III)Cbi^{2+} species. Addition of py to such a (disolvato)Co(III)Cbi^{2+} solution produced pronounced spectral changes (including shift of the γ band from 349 to 361 nm, Figure 8) compared to the changes that occur when py binds to Co(II)Cbi$^+$ (Figure 7a). Also noticeable in Figure 8 is a distinct α band[35] (containing three individual maxima) between 450 and 550 nm. These changes all indicate axial binding of the N-donor py ligand to (disolvato)Co(III)Cbi^{2+} in ethylene glycol.[35] No spectral changes and hence no binding were observed upon addition of highly hindered ligands (2-pic and 2,6-lut, spectra not shown).

Discussion

Methodology for Assessing Axial Ligand Binding to Co(II)Cbi$^+$. As we shall establish below, numerous misleading observations in the literature arise from the weak and labile axial ligand binding, solubility only in coordinating solvents, and air sensitivity of Co(II)Cbi$^+$. We believe the results presented here now provide a definitive evaluation of the solution species. Since our results contradict observations and conclusions in the literature and depend on the methodology we employed, we first discuss our methods.

A Combined Spectroscopic Assessment of Axial Ligand Binding to Co(II)Cbi$^+$. The combination of visible absorption and EPR spectroscopy provides both complementary and reinforcing information. EPR spectroscopy is selective because only the Co(II) state is paramagnetic. EPR spectroscopy is a definitive technique for assessing binding of low spin Co(II) to nitrogenous ligands, since superhyperfine coupling between the unpaired electron on Co(II) and the $I = 1$ ^{14}N nucleus will result only when binding occurs. In addition, since axial ligand binding is fast, the low-temperature solution conditions achieved prior to formation of frozen glasses used in EPR measurements favor binding by exogenous ligands.[41,44] Our EPR experiments, performed on glasses at 120 K, have shown that all of the Co(II)Cbi$^+$ is bound in the presence of only 5 mM py in a mainly noncoordinating solvent (2-pic) and that almost all is bound in the presence of 10 mM py in a coordinating solvent (ethylene glycol, Figure 2a). In contrast, visible spectral changes indicate that full formation of pyCo(II)Cbi$^+$ does not occur at room temperature until py concentrations reach ~0.50 M (Figure 7a, inset). *Thus, the EPR experiment provides a sensitive assessment of ligand binding even when there is little binding under ambient conditions.*

Visible absorption spectra contain contributions from all cobinamide species present in the solution, regardless of the cobalt oxidation state. Any binding by an axial ligand would change the visible spectrum, even if the axially bound ligand changed the spin state of Co(II) or promoted the oxidation of Co(II) to Co(III). In either case, no EPR signal would be observed for the adduct (EPR signals of high spin Co(II) are usually not detected at 120 K, and Co(III) is diamagnetic). Either type of species would decrease the low-spin EPR signal intensity, but this decrease would be difficult to detect if only a low percentage of high spin Co(II) or Co(III) species were formed. In contrast, changes in intensity are easily detected in visible spectra. Many of the visible spectra depicted in a previous study on the binding of sterically hindered pyridine derivatives to Co(II)Cbi$^+$ in ethylene glycol[22] are very similar to those we observe (cf. Figure 8) for the binding of unhindered pyridine ligands to (disolvato)Co(III)Cbi^{2+}. The reported spectra do not resemble spectra characteristic of LCo(II)Cbi$^+$ adducts. Our findings demonstrate that the visible spectral changes attributed to axial ligand binding of both unhindered and hindered N-donor ligands to Co(II)Cbi$^+$ were misinterpreted in several cases and that the reported changes are due mainly to Co(III) species.[22] EPR spectra of Co(II)Cbi$^+$ bound to dioxygen show a characteristically strong signal splitting near $g = 2$.[29] This signal helps to define conditions and methods that exclude dioxygen. In addition to excluding dioxygen, the possibility that the ligand binding to Co(II)Cbi$^+$ is an impurity should be excluded by (a) utilizing at least two suppliers of the ligand, (b) varying the concentration of the complex, and, if doubt persists, (c) seeking alternative ligand purification methods.

Development of a Co(III)-Affinity Distillation Technique to Selectively Remove Trace Impurities in Liquid Nitrogenous Ligands. The Co(III)-affinity distillation technique we have developed here proved to be a much more effective method than regular distillation to remove trace impurities present in commercial 2-pic. It is known that commercial 2,6-lut and 2,4,6-collidine can be contaminated with impurities.[40,41] The distillation technique that has generally been used to remove such impurities involves addition of BF_3-etherate to the ligand.[40,41] However, BF_3-etherate is corrosive and toxic, and the distillation procedure is somewhat complicated.[40,41] Our much simpler technique utilizes an easily synthesized Co(III) complex, Co((DO)(DOH)Me$_2$pn)Br$_2$ (Chart 1). Addition of this complex before distillation of 2-pic at low temperature removes unhindered ligating impurities from 2-pic. A red solution of the dark green Co(III) complex indicates the presence of impurities. After the level of impurities is reduced, the color remains green.

Correlation between Axial Ligand pK_a and Co(II) Hyperfine Coupling Constant. Employing the methods just described, we obtained EPR data establishing a linear increase of the Co(II) hyperfine splitting of 4-X-pyCo(II)Cbi$^+$ with a decrease of the 4-X-py pK_a value (Figure 3). The hyperfine coupling is governed by the overlap of the sp^2(N) + $3d_{z^2}$ (Co(II)) σ molecular orbitals.[45] The Co−N σ-bonding molecular orbital with two paired electrons resides largely on Co, and the corresponding σ*-antibonding orbital containing the unpaired electron resides largely on N.[45,46] The size of the N lone pair

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(44) Alston, K.; Storm, C. B. *Biochemistry* **1979**, *18*, 4292−4300.

(45) Lee, H. C.; Scheuring, E.; Peisach, J.; Chance, M. R. *J. Am. Chem. Soc.* **1997**, *119*, 12201−12209.

(46) Arcos, T.; de Castro, B.; Ferreira, M. J.; Rangel, M.; Raynor, J. B. *J. Chem. Soc., Dalton Trans.* **1994**, 369−377.

orbital decreases as ligand pK_a decreases, making overlap of the N lone pair and the Co $3d_{z^2}$ orbital less effective for bonding.[46] The resulting increase in the contribution of the Co(II) $3d_{z^2}$ orbital to the σ^*-orbital increases the amount of unpaired spin density on Co(II) and therefore increases the hyperfine interaction.[45,46] This analysis explains the increase in the Co(II) hyperfine splitting as 4-X-py ligand basicity decreases. The finding that the smallest and largest hyperfine splittings were observed for 4-Me_2NpyCo(II)Cbi$^+$ and 4-CNpyCo(II)Cbi$^+$, respectively, indicates that the unpaired electron spin on Co(II) is most and least delocalized onto the bound 4-Me_2Npy and 4-CNpy, respectively. This is the relationship expected from the basicities of these similarly sized ligands.

On the Question of the Binding of Ligands Requiring Formation of Hyper-Long Bonds to Co. In an early study, EPR spectroscopic results with both 2-pic and 2,6-lut were interpreted to suggest that steric factors hindered formation of LCo(II)Cbi$^+$, but once it was formed, no weakening in Co−N bond strength was indicated by the measured hyperfine splittings, which were very similar to that of pyCo(II)Cbi$^+$.[24] Inspection of models suggested that quinoline ligands would have unfavorable steric interactions with the corrin (studies on Co(II) porphyrins indicated that quinoline is a weak ligand).[47] In addition, X-ray structural analysis of a 4-methylquinoline Co synthetic model reveals that the Co−N bond length is ∼0.12 Å longer than in the unhindered pyridine analogues (unpublished studies). Similar complexes with the 2-vinylpy and 2-pic ligands have only slightly shorter Co−N axial bonds than that of the 4-methylquinoline complex. The overlap of the N lone pair and the Co $3d_{z^2}$ orbital becomes less effective with increased ligand steric bulk due to the longer Co−N bond. Thus, it would be expected that an adduct of a sterically bulky ligand, which would form a complex with an elongated Co−N axial bond, should have a larger hyperfine splitting. The EPR signals observed here at ≥8 M unpurified 2-pic (Figures 5a and 6a) were strong enough to permit measurement of an ∼10.95 mT Co(II) hyperfine splitting, a value similar to that of pyCo(II)Cbi$^+$ (11.04 mT). Thus, our initial experiments gave misleading results similar to those found by the earlier investigators. In the presence of a high concentration (8−9.2 M) of highly purified 2-pic, no signals for a LCo(II)Cbi$^+$ species were detected, and the Co(II) hyperfine splitting of unbound Co(II)Cbi$^+$ alone was observed. The hyperfine octet features were not broader than those measured in the absence of any added N-donor ligand, a result which eliminates the possibility of even a very long Co−N bond with 2-pic.

Visible spectroscopic changes indicating binding to Co(II)Cbi$^+$ in ethylene glycol were evident at 0.005 M py and were complete by ∼0.50 M py (Figure 7a). The absence of change in the visible spectrum of Co(II)Cbi$^+$ on addition of up to 2 M 2-pic showed that no binding occurred (Figure 7b). This result is consistent with the results of our EPR experiments in the presence of 2-pic purified by Co(III)-affinity distillation.

We were able to obtain visible spectral effects similar to those in the literature reports claiming formation of Co(II)Cbi$^+$ adducts with long Co−N bonds[22] *only* if we (a) allowed ambient oxygen into the system during or after photolysis of MeCbi$^+$ *and* (b) used an unhindered pyridine ligand, as shown in Figure 8. A logical conclusion from our work is that the cobinamide had been partially oxidized in some experiments reported in ref 22.

We can rank the ability of the N-donor ligands to bind to Co(II)Cbi$^+$ as follows: 4-Me_2Npy > 4-pic > py > 4-CNpy >>> any quinoline or any 2-X-py. The equatorial plane of the cobinamide apparently provides too much steric bulk to allow any of the 2-X-py's to bind. Our results prove beyond question that 2-pic itself does not bind to Co(II)Cbi$^+$.

Implications of Our Results. Previous Kinetic and Axial Ligand Binding Studies with Cobinamides Employing Visible Spectroscopy. The recent study proposing long bonds[22] also presented evidence that sterically hindered ligands such as 2-pic and 2,6-lut increase the rate of Co−C bond cleavage of adenosylcobinamide (AdoCbi$^+$, Figure 1) in ethylene glycol at 110 °C. Visible spectra obtained at 25 °C led the investigators to conclude that neither ligand bound detectably to AdoCbi$^+$ but that both did bind to Co(II)Cbi$^+$ at this temperature. Other observations by these authors appeared to indicate that the relative amount of Co−C bond heterolysis versus homolysis products at 110 °C was dependent on the presence of these ligands. Also, molecular mechanics calculations performed by the investigators supported the feasibility of the formation of cobinamides with 2-pic and 2,6-lut bound at an axial site by a very long Co−N bond (∼0.1 Å longer than normal). These authors used their evidence for bulky ligand binding to Co(II)Cbi$^+$, but not AdoCbi$^+$, to conclude that such bulky ligands could stabilize the Co(II) state and hence the transition state. This state was proposed to contain {[Ado$^\bullet$- - -$^\bullet$CoCbi·bulky base]$^+$}‡ as the activated complex, a proposition supported by the apparent dependence of the cleavage rate on the concentration of the bulky ligands. Invoking the principle that such a kinetic result "reveals the composition of the activated complex of the rate-determining step",[22] the authors concluded that the bulky ligand "must be involved in the rate-determining Co−C bond cleavage step".[22] The general conclusions reached were that the role of the axial ligand was most important in the transition state and that the Co center became Co(II)-like in the transition state.

We believe the conclusions described in the preceding paragraph cannot be accepted as being correct. In particular, we cite our visible and EPR data demonstrating that 2-pic and 2,6-lut do not bind. We attribute the reported observations to impurities in the two ligands.[22] Furthermore, other experimental problems are also evident in the published study.[22] In all reported titrations involving pyridine-type ligands, the spectral traces did intersect but did not define an isosbestic point.[22] The failure to observe an isosbestic point was attributed to a mixture of axial ligation at both the α- and β sides of the corrin ring of Co(II)Cbi$^+$.[22] However, such a rapidly equilibrating equilibrium mixture must give an isosbestic point. Addition of py to Co(II)Cbi$^+$ gave isosbestic points only when stringent efforts were made to exclude dioxygen (Figure 7a and Supporting Information). The visible spectral traces reported for "Co(II)Cbi$^+$" binding to 2,6-lut (Figure 5 of ref 22) and to 3-pic (Supporting Information of ref 22) have features similar to those found for unhindered pyridine-type ligand binding to oxidized Co(II)Cbi$^+$ (Figure 8).

Previous EPR Studies of Cobinamides. Our results demonstrating that several previous observations are erroneous remove an important experimental obstacle to the application of aspects of EPR theory to B_{12} enzymes. The 2-picCo(II)Cbi$^+$ adduct does not exist at a detectable level, and thus, there is no longer an example of a cobinamide model with a long axial Co−N bond but a normal hyperfine splitting.[24]

Influence of Protein on Co(II) Hyperfine Splitting in Enzyme-Bound Cofactors. The Co(II) hyperfine splitting in cofactor-enzyme complexes has been determined for five B_{12} enzymes. No significant difference in hyperfine splitting from the free cofactor was found in EPR studies with three of the

(47) Walker, F. A. *J. Am. Chem. Soc.* **1973**, *95*, 1150−1153.

B_{12} enzymes. This group of enzymes includes ribonucleotide triphosphate reductase[48] and ethanolamine deaminase,[49] which are ligated by DMBz, and 2-methylene glutarate mutase, which is ligated by a histidine Imd.[50] The absence of a detectable protein-induced change in hyperfine splitting in 2-methylene glutarate mutase[50] agrees with the free-solution Co−N bond length calculated from EXAFS data.[15]

For the other two of these five B_{12} enzymes, the X-ray crystallographic structure has been determined. In methylmalonyl-CoA mutase with axial histidine Imd ligation, the hyperfine splitting is 10.8 mT,[51] whereas the splitting for imidazoleCo(II)Cbi$^+$ in methanol glass is 11.1 mT.[23] In diol dehydrase with axial DMBz ligation, the hyperfine splitting is 10.5 mT,[7,49] compared with the value of 11.0 mT for cob(II)alamin in a variety of different solvents.[23] The decreases by 0.3−0.5 mT in hyperfine splitting for the bound cofactor indicate that there is a stronger overlap of the axial N and Co orbitals.[46] Stronger orbital overlap could arise from a shortened Co−N bond length in situ, relative to the free cofactor. This assessment of the effect of protein binding on in situ Co−N bond length contrasts with reports, based on X-ray crystallographic[11,14] and EXAFS[52] studies, of hyper-long Co−N bonds in these two holoenzymes. Heterogeneities in (Co(II)/Co(III)) redox state and Co(III) sixth axial ligand position have been proposed[15] to interfere with the X-ray crystallographic determinations of the Co−N distances in methylmalonyl-CoA mutase and diol dehydrase. These problems do not plague the EPR results, however, since EPR spectroscopy is sensitive only to the paramagnetic Co(II) form of the cofactor. Thus, our work adds support to a previous assessment that the Co−N bond length is not distorted in those forms of the enzyme studied thus far.[53]

Conclusions

The combination of EPR spectroscopy and visible spectroscopy is a powerful approach for assessing the binding of pyridine derivatives to Co(II)Cbi$^+$. A linear correlation exists between the Co(II) hyperfine splitting of 4-X-pyCo(II)Cbi$^+$ and the pK_a value of the unhindered 4-X-pyridine ligands. This hyperfine splitting increased with a decrease in axial ligand basicity as predicted by theory. Previous literature studies contain erroneous evidence of bulky pyridine ligand binding to Co(II)Cbi$^+$.[22−24] The adducts observed were actually formed by nitrogenous impurities present in commercial supplies, even after standard distillation. Using 2-pic as an example, we have shown that these impurities can be removed by our new Co(III)-affinity distillation technique. EPR spectra of Co(II)Cbi$^+$ in the presence of purified 2-pic are essentially identical in intensity, shape, and line width to those of solvated Co(II)Cbi$^+$ in ethylene glycol. Artifacts caused by such impurities have also confounded results of model experiments devoted to assessing the mechanism of the Co−C cleavage step.[22] In particular, the evidence that the influence of the axial ligand occurs primarily in a Co(II)-like activated complex is now in doubt. We believe that kinetic studies combined with careful scrutiny of the observable forms by all feasible spectroscopic methods will eventually yield the most complete insight into mechanism.

Special care is needed in studies of cobinamide models since these B_{12} derivatives appear to be particularly selective in binding to sterically unhindered sp^2 N-donor ligands. Since the cobinamide model proves to have a high degree of steric demand and since all 2-substituents on pyridine studied prevent detectable binding, we are presently evaluating both synthetic Co(II) models and other classes of sterically hindered N-donor ligands. We seek adducts useful for experimentally assessing the relationship between Co(II) hyperfine splitting and Co−N bond length.

Finally, our results resolve the conflict between EPR experiment and theory. At this time, the reported hyperfine splitting in B_{12} enzymes leads most reasonably to the conclusion that the Co(II)−N axial bond is not distorted from that in unbound cofactors. We agree with the previous suggestion[53] that such an interpretation does not exclude the possibility that the bond is lengthened in the bound cofactor for other forms of the enzyme. For example, a long Co−N bond is still possible at an intermediate or transition state in the catalytic cycle.

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Note Added in Proof. A correction to ref 22 has recently appeared (Sirovatka, J. M.; Finke, R. G. *Inorg. Chem.* **2001**, *40*, 1082). The authors now recognize the problem of oxidation of Co(II)Cbi$^+$ to Co(III)Cbi^{2+} in their experiments. However, as yet, they do not recognize the presence of ligand impurities and do not withdraw their major conclusions. As stated above, we believe the conclusions in ref 22 cannot be accepted as being correct. In fact, our work confirms the presence of ligand impurities and the absence of binding by sterically hindered bases, placing the conclusions in ref 22 in serious doubt.

Supporting Information Available: Full EPR spectra of Co(II)Cbi$^+$ with unpurified and purified 2-picoline; expanded visible spectra of Co(II)Cbi$^+$ titrated with pyridine and of (disolvato)Co(III)Cbi^{2+} with CN^- (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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