Near-IR FT-Raman Spectroscopy of Methyl-B_{12} and Other Cobalamins and of Imidazole and Imidazolate Methylcobinamide Derivatives in Aqueous Solution

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Near-IR FT-Raman spectra of B_{12} imidazole derivatives and cobalamins in aqueous solution were compared since there is now strong evidence that, in human B_{12}-dependent enzymes, the 5,6-dimethylbenzimidazole (DMBz) is replaced by imidazole from a histidine in the protein. Derivatives studied include methylcobalamin [MeCbl (DMBz base-on) and $MeCbl^+$ (base-off by acidification to protonate the DMBz)], methylaquacobinamide (Me-Cbi^+) [Cbi's have the DMBz-bearing nucleotide loop removed by hydrolysis], and Me(*N*-acetylhistidine)Cbi [coordinated through imidazole in Me(*N*-AcHis)Cbi at pH 10 and imidazolate in Me(*N*-AcHis)Cbi^- in 1 M NaOH]. Several marker bands changed with changes in the axial ligand *trans* to the methyl group. The *frequency* of the Co−CH_3 stretching mode at ∼505 cm^{-1} (assigned by isotopic shift using −CD_3) was similar for all MeCbl and MeCbi species; thus, the *trans* ligand, including the very powerful electron-donating imidazolate species, has little effect on Co−C bond strength. In contrast, the *peak height* of the Co−CH_3 band, relative to the corrin long-axis mode band at 1495 cm^{-1}, consistently increased 2-fold upon coordination of an N-donor ligand to $MeCbi^+$ and $MeCbl^+$. This intensity change appears to be a useful means of assessing ligand replacement reactions. With increasing *trans* ligand donor ability, the frequency of the corrin in-phase double-bond stretching mode along the short axis at ∼1545 cm^{-1} increased, but the frequencies of the corresponding long-axis mode at ∼1495 cm^{-1} and the corrin band at ∼1570 cm^{-1} were unchanged. The frequency of a corrin band at ∼1600 cm^{-1} increased slightly to 1603 cm^{-1} from the base-on to the base-off MeCbl form; the band is also at 1603 cm^{-1} for $MeCbi^+$, Me(*N*-AcHis)Cbi, and Me(*N*-AcHis)Cbi^-. A decrease in the frequency of the ∼1570 cm^{-1} band coincident with acid-catalyzed H-to-D exchange at the corrin C10 in acidic D_2O solutions (confirmed by ^1H NMR spectroscopy) was evidence that it is a corrin band. However, the frequency and intensity of this band were relatively insensitive to the *trans* ligand. This diverse dependence of the corrin-band frequencies on changes in *trans* axial ligand bulk suggests that in these B_{12} derivatives there are no large structural distortions. Differences in electron donation by the *trans* ligands have the greater influence on the spectra. A band at ∼1315 cm^{-1} was found to be characteristic of unprotonated DMBz; it is absent when DMBz is removed (Cbi's) or protonated ($MeCbl^+$) but present in all other Cbl's, including base-off $(CN)_2Cbl^-$. An ∼30 cm^{-1} shift to lower frequency of the overlapping amide I bands of cobalt corrinoids between H_2O and D_2O was caused by amide NH_2 to ND_2 exchange. The frequency shift of ∼10 cm^{-1} of this band between H_2O and ethanol was consistent with a small redistribution of resonance forms of the amide group between solvents. Since the band for the CD_3 symmetric stretch of the Co−CD_3 group lies in a region of the spectrum (∼2105 cm^{-1}) that is devoid of other bands, it may be useful in studies of enzyme-bound Me-d_3-Cbl. In summary, our results show that the exchange of DMBz by imidazole has minimal influence on the methylcobalt(III) ground state. We suggest that the functional role of the imidazole occurs later in the catalytic cycles of B_{12} enzymes.

Introduction

In humans, two enzymes, methionine synthase[1] and methylmalonyl-CoA mutase,[2] are known to be dependent on organocobalamins as cofactors. While methionine synthase catalyzes a methyl group transfer in a process involving heterolytic cleavage of a cobalt−carbon bond, methylmalonyl-CoA mutase catalyzes a carbon skeleton rearrangement[3] of the substrate to form the product in a process involving homolytic cleavage. For methionine synthase, the prosthetic group is methylcobalamin (MeCbl), consisting of a central cobalt ion chelated by a corrin ring and containing a methyl group bound to cobalt on the "upper" β side (Figure 1). Spectroscopic probes may be useful in studying the effects of the environment on the Co−CH_3 bond[4] and may be extended to the enzyme systems, in particular methionine synthase, in which the methyl group is protected from solvent and is buried in a very hydrophobic region.[4] N3 of the 5,6-dimethylbenzimidazole (DMBz) base is positioned for coordination on the "lower" α side at neutral to high pH (MeCbl in the base-on form) because the base is connected to the corrin by a nucleotide loop. The plane of the base lies along the "long" axis, i.e. C5−Co−C15. The corrin is bent upward in a butterfly manner with the "body" of the butterfly lying along the short axis (C10−Co−midpoint of the C1−C19 bond). At low pH, the base-off species, $MeCbl^+$, is formed (apparent pK_a[5] of the protonated DMBz N3 is 2.9).

The recent crystal structure determination of MeCbl bound to the cobalamin-binding domain of *Escherichia coli* methionine synthase[4] has revealed much new information. Specifically, the MeCbl was found to be in the base-off conformation, with the "nucleotide loop" important primarily in the binding of the

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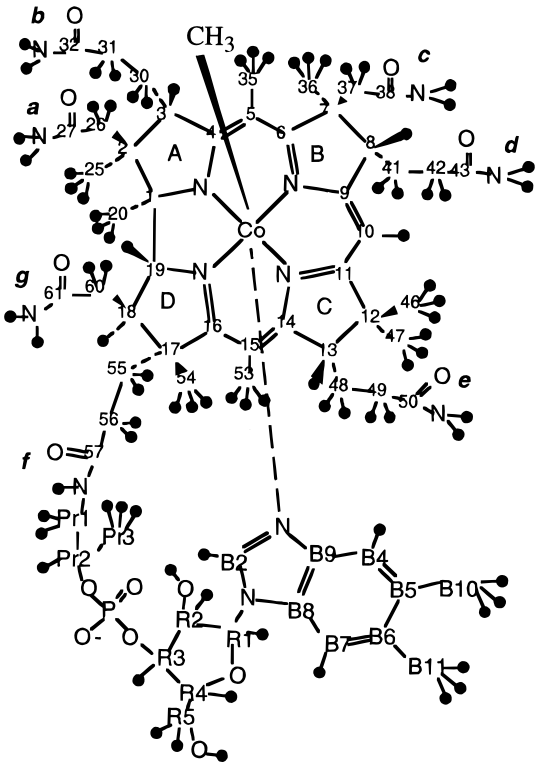

Figure 1. Structure, numbering of atoms, and designations of pyrrole rings of methylcobalamin (MeCbl).

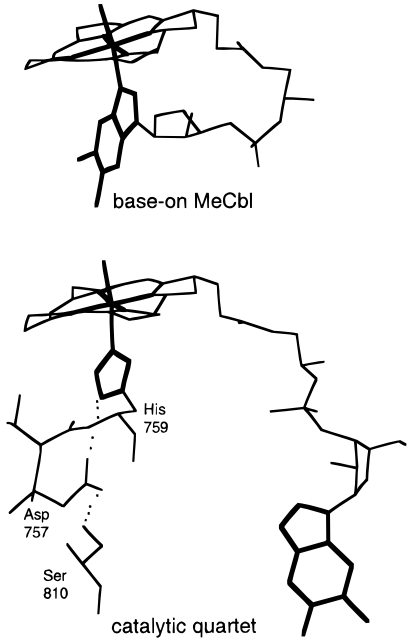

Figure 2. Comparison of the structures of MeCbl in the base-on form[12] and in the B_{12} binding domain of methionine synthase.[4]

cobalamin to the protein (Figure 2). Also, a histidine (His^{759} in the enzyme) is coordinated at the α-axial site. This residue has been postulated to be part of a "catalytic quartet" involving the Cbl, His^{759}, Asp^{757}, and Ser^{810}. In this hypothesis, a hydrogen-bonding network connects the four components of the quartet. The Asp^{757} carboxylate attracts the imidazole proton of His^{759}, weakening the NH bond. This interaction gives the imidazole imidazolate character, making it a better ligand, which stabilizes the hexacoordinate methylcob(III)alamin species. In other phases of the catalytic cycle, proton uptake by the quartet makes imidazole a weaker ligand and thus favors the square-planar cob(I)alamin (B_{12s}) species. Such stability would facilitate transfer of the methyl group to homocysteine in the methionine-forming step. Recent EPR evidence has shown that a nitrogenous ligand (probably the imidazole of an enzyme histidine residue) displaces the DMBz moiety of coenzyme B_{12} in *Propionibacterium shermanii* methylmalonyl-CoA mutase,[2,6] suggesting that this may be a common motif in cobalamin-dependent enzymes. Spectroscopic methods for assessing the form of the bound cobalamins are needed.[2]

The Co−CH_3 stretching vibrational frequency can be correlated to Co−C bond strength in the ground state, but except in rare cases[7] this band is difficult to observe by the usual types of Raman spectroscopy because the organocobalt species are light sensitive. Recent elegant studies have shown that resonance Raman spectroscopy is a powerful way to elucidate the nature of the key M−C bonds in carbon monoxide dehydrogenase.[7] Early studies utilizing near-IR FT-Raman spectroscopy[8] showed for the first time that the Co−CH_3 stretching band is observable for solid-state MeCbl and organocobalt B_{12} models.[9,10] The relatively low-excitation-energy (1.064 μm) Nd-YAG laser used in this technique does not promote electronic transitions, thus eliminating potential problems associated with fluorescence and photolysis of the Co−C bond.[8]

An in-depth FT-Raman spectral study of cobaloxime B_{12} models ($LCo(DH)_2CH_3$), where L is a neutral, monodentate ligand and DH is the monoanion of dimethylglyoxime, showed that the Co−CH_3 stretching frequency was sensitive to the steric bulk of L (steric *trans* influence) rather than to the electronic *trans* influence.[10] The Co−C stretching band, *trans* axial ligand bands, and equatorial ligand bands underwent significant shifts between solution and solid states. Furthermore, the intensity of the Co−C stretching band in B_{12} model compounds was found to be sensitive to the nature of the *trans* ligand. An ∼6-fold increase in the Raman scattering intensity of the Co−C mode has been observed in methylcobaloximes[9] upon going from $H_2OCo(DH)_2CH_3$ to $(PPh_3)Co(DH)_2CH_3$. Similarly, an increase of ∼10-fold was reported in the same mode when L was changed from H_2O to trimethylphosphine or 4-*tert*-butylpyridine in [LCo(bpb)CH_3], where bpb = 1,2-bis(2-pyridinecarboxamido)benzene.[11]

In this work, a series of cobalt corrinoids were investigated by FT-Raman spectroscopy. Biologically relevant aqueous solution conditions were employed. The FT-Raman technique permitted data collection at frequencies <700 cm^{-1}, which would prove extremely difficult in aqueous solution using FT-IR. In addition, the instrument employed in the current set of experiments allowed measurements at frequencies lower than those used in previous FT-Raman studies.

We wanted to determine if the spectra, particularly the Co−C stretching band, were sensitive to substitution of the *trans* ligand as found for models, since such substitution occurs in the binding of the cofactor to human B_{12} enzymes. Methylcobinamide ($MeCbi^+$) derivatives are well suited for such studies. MeCbi's contain the important corrin ring and the axial methyl group but lack the nucleotide loop; this loop favors DMBz coordination and thereby inhibits coordination by other ligands. The DMBz protonation needed in nonenzymic systems to deligate the DMBz requires acid conditions that also prevent coordination by imidazole ligands. Thus, $MeCbi^+$ will add a histidine ligand,

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producing a species that is a good initial structural model of the Co coordination environment in the enzyme system. Spectroscopic assessment of the Co−C bond strength of methyl(*N*-acetylhistidine)cobinamide (Me(*N*-AcHis)Cbi) at high pH, where the imidazolate form is bound, allows us to evaluate the hypothesis that an imidazolate or imidazolate-like species weakens the Co−C bond.

Experimental Section

MeCbl was prepared following a previously described method[12] except that a Pharmacia XK 26/40 column attached to a Pharmacia FPLC system and packed with 32 cm of SP-Sephadex C-25 was used. Fractions were monitored at 365 nm. Those fractions with UV/vis spectra consistent with MeCbl were pooled; then MeCbl was crystallized by addition of acetone. The long, deep-red crystals were checked for purity by 360 MHz NMR in D_2O. Me-d_3-Cbl was prepared similarly, using methyl-d_3 iodide (Cambridge Isotopes, 99.5%). Base-off $MeCbl^+$ was prepared by dissolving MeCbl in 500 mM HCl. MeCbl-*10-d* was prepared by dissolving MeCbl in D_2O acidified to 500 mM HCl with concentrated HCl and then lyophilizing the sample in a Savant Speed Vac.

Methylcobinamide acetate (MeCbiOAc) was prepared by $Ce(OH)_3$-catalyzed hydrolysis.[13] $MeCbi^+$ and the CD_3 analog (phosphate forms) were prepared in 25% yield by using the method to prepare factor B[14] as follows: MeCbl (50−500 mg) was stirred for 7−10 days with CF_3SO_3H (1−10 g, Aldrich) at room temperature. Reactions were monitored by following the emergence of the 461 nm ($MeCbi^+$) band under conditions that ensure MeCbl is base-on (detection by 522 nm band): aliquots (2−5 μL) were diluted into 10 mM NaOH so as to give an absorbance <2. A Pharmacia 5 mL HiTrap SP column was used in an FPLC ion-exchange step. Counterion exchange of $MeCbi^+$ was also accomplished with this column to obtain the chloride form, MeCbiCl. After the solutions were desalted on a Pharmacia XK 26/20 column packed with 10 cm of Bio-Beads SM-2 adsorbant (Bio-Rad, analytical grade, 100−200 mesh), the sample was eluted with 50% acetonitrile and the solvent was removed. The resulting powders were assessed by ^{1}H NMR and vis spectroscopy. The MeCbiCl and MeCbiOAc forms had the same spectra, and only data for the former are reported here.

Possible chloride-binding to $MeCbl^+$ and to MeCbiCl in 500 mM HCl was checked by comparing the visible spectra (350−800 nm) of MeCbiCl (0.6 mM) solutions in water, in 500 mM HCl, in 250 mM H_2SO_4, and in 500 mM HNO_3; no differences were seen.

The excess of *N*-acetylhistidine needed to form Me(*N*-AcHis)Cbi species was determined by progressively increasing the concentration of ligand in $MeCbi^+$ solutions (317 μM, based[15] on $\epsilon^{461nm} = 10{,}700$ M^{-1} cm^{-1}) at pH 10 and in 1 M NaOH until there were no changes in the UV/vis spectrum. A quartz cuvette with a 1 mm path length was used to allow maximum $MeCbi^+$ concentrations while keeping the absorbance below unity (at pH 10.2, $\epsilon^{525nm} = 10\,200$ M^{-1} cm^{-1}, and in 1 M NaOH, $\epsilon^{536nm} = 8400$ M^{-1} cm^{-1}). The required excess concentration of ligand determined in this manner was lower (0.71 M) in 1 M NaOH than at pH 10 (1.91 M) since imidazolate is a better ligand. These were the minimum excess concentrations used in all experiments. For FT-Raman experiments, for example, where Co concentrations were often 50−100 mM, the total ligand concentrations were ∼2 M at pH 10. Concentrated (3 M) stock solutions of Na(*N*-AcHis) were prepared by first neutralizing the free acid (Aldrich) with equimolar NaOH and then adjusting the pH to 10 or by adding an excess for a 1 M hydroxide solution. The UV/vis spectrum of $MeCbi^+$ in the absence of ligand was checked in these two high-pH solutions, and no immediate changes were seen. Slight changes in the UV/vis spectrum upon prolonged exposure to high pH were consistent with limited hydrolysis of the amide side chains to an extent not detectable by FT-Raman spectroscopy. Dicyanocobalamin was prepared as described.[16] Dicyanocobinamide (Sigma) was dissolved for Raman experiments without an excess of cyanide after it was found that the UV/vis spectrum did not change in the presence of 550 mM KCN, pH 11.

Methyl(imidazole)cobinamide ($Me(Imd)Cbi^+$), methyl(imidazolato)-cobinamide ($Me(Imd^-)Cbi$), and methyl(*N*-methylimidazole)cobinamide ($Me(N\text{-}MeImd)Cbi^+$) were prepared from MeCbiCl (0.5 mM) and 5 M ligand in 40% methanol 1 M in $NaNO_3$. UV/vis spectra were recorded ($A < 2$) in a 1 mm quartz cuvette.

UV/vis spectra were recorded on a Varian Cary 3 UV/vis spectrophotometer. FT-Raman data were collected on a Nicolet Raman 950 instrument, using the liquid-nitrogen-cooled germanium detector supplied with it, 4 cm^{-1} resolution, a CaF_2 beam splitter, a mirror velocity of 0.4747 cm/s, and power settings (at the sample) of 16−71 mW for the solids (1000−4864 scans depending on stability in the laser), and 1−1.5 W for solutions (1000−10 000 scans depending on concentration). Typical corrinoid concentrations ranged from 15−20 mM for the base-on Cbl's to 60−100 mM for the Cbi's and base-off Cbl's. Solvent spectra were subtracted, using the disappearance of the water band at ∼450 cm^{-1} as an indication that subtraction was complete. When an excess of ligand was used, spectra of free-ligand solutions at the correct excess concentrations were subtracted from the spectra of the complexes. Intensities were normalized with respect to the band at ∼1495 cm^{-1} as an internal standard since its intensity remains relatively constant with pH above pH 4.[17] In all of the corrinoid spectra, the band at ∼1495 cm^{-1} is the most intense.

Results and Discussion

Co−CH_3 Stretch, ∼505 cm^{-1}. The frequencies and intensities of the Co−CH_3 stretching mode of various methylcobalt(III) corrinoids are compared in Table 1. The Co−CH_3 stretching frequency, assigned by isotopic substitution at the methyl group (Figure 3), depended little on the nature of the axial *trans* ligand. However, the intensity of the band is a good indicator of whether or not an N-base was bound to the cobalt. Compared to the case of an aqua ligand coordinated *trans* to the methyl group, the relative intensity was approximately twice as great for DMBz, *N*-$AcHis^-$, or *N*-$AcHis^{2-}$ (Figure 4). The ratio of the peak height (H) of the ∼505 cm^{-1} band to that of the ∼1495 cm^{-1} band (H_{505}/H_{1495}) is significantly different for the five base-off/Cbi forms [0.092 ± 0.004 (σ_{n-1})] compared to the six forms with an N-donor [0.20 ± 0.02]. Relative peak heights of Co−CH_3 bands are given in Table 1. (The higher intensity of the Co−CD_3 compared to the Co−CH_3 band in Figure 3 is caused by overlap with another band at 475 cm^{-1}.)

We wanted to confirm the formation of the imidazole and imidazolate species using UV/vis spectroscopy. The changes in the Raman spectrum of $MeCbi^+$ were accompanied by changes in the visible spectrum of the cobinamide that included a shift in λ_{max} from 461 nm ($MeCbi^+$) to 527 nm [Me(*N*-AcHis)-Cbi at pH 10] and to 536 nm [Me(*N*-AcHis)Cbi^- in 1 M NaOH]. Although red shifts similar to the latter change have been attributed to imidazole deprotonation in imidazole complexes of B_{12} derivatives lacking Co−alkyl carbon bonds,[18,19] none have been reported for organocobalt corrinoids. To determine if the red shift at high pH resulted from formation of the imidazolate species, a similar experiment was performed with $Me(Imd)Cbi^+$, which has an ionizable proton, and $Me(N\text{-}MeImd)Cbi^+$, which does not. In 1 M NaOH, the red shift seen in Me(*N*-AcHis)Cbi was observed with $Me(Imd)Cbi^+$ but not with $Me(N\text{-}MeImd)Cbi^+$. The 527 nm peak in the UV/vis spectrum of $Me(Imd)Cbi^+$ shifted to 538 nm. Thus, we attribute

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Table 1. Selected FT Raman Spectral Bands (cm^{-1}) of B_{12} Derivatives in Solution

derivative[a]	solvent	amide I	corrin	corrin	corrin short axis	corrin long axis	DMBz	Co−CH_3 (rel peak height)[b]
MeCbl	H_2O, pH 9.3	1670	1600	1571	1544	1494	1312	504 (2.2)
MeCbl	D_2O, pH 10	1639	1600	1570	1543	1494	1314	506 (1.7)
MeCbl-*d*	H_2O, pH 9.4	1668	1599	1564	1537	1493	1312	505 (2.1)
MeCbl	ethanol	1680	1599	1569	1544	1494	1316	503 (2.4)
$MeCbl^+$	500 mM HCl	1670	1603	1573	1538	1494		506 (1.1)
$MeCbl^+$	500 mM HCl/D_2O	1641	1602	1565	1534	1492		506 (1.0)
$MeCbi^+$	500 mM HCl/D_2O	1641	1602	1565	1534	1492		504 (1.0)
$MeCbi^+$	D_2O, pH 6.5	1640	1604	1575	1540	1495		506 (1.0)
$MeCbi^+$	H_2O, pH 7.7	1672	1603	1573	1538	1494		504 (1.0)
Me(*N*-AcHis)Cbi	H_2O, pH 10	1674	1603	1573	1548	1495		506 (2.3)
Me(*N*-AcHis)Cbi^-	1M NaOH	1663	1603	1572	1549	1495		506 (2.2)
CNCbl	H_2O, pH 9	1665	1604	1578	1546	1500	1318	
$(CN)_2Cbl^-$	H_2O, pH 9	1675	1616	1584	1556	1506	1310	
$(CN)_2Cbi$	H_2O, pH 8	1670	1616	1583	1556	1506		

[a] Conditions: ~15−100 mM, 1−1.5 W laser power, and 1000−10000 scans. [b] *H*, calculated by dividing the amplitude of each Co−CH_3 band by the amplitude of the band at 1495 cm^{-1} and normalizing to the base-off values of unity.

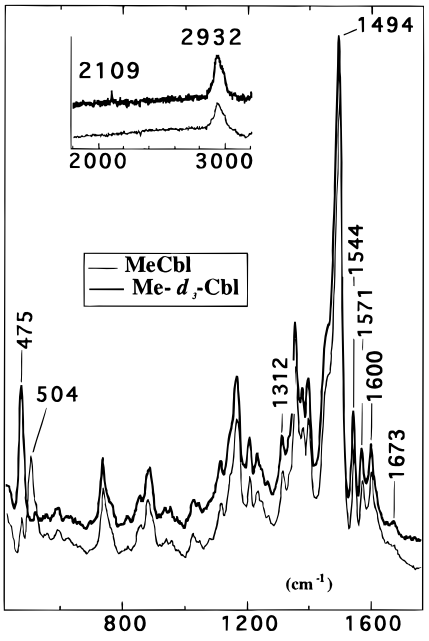

Figure 3. FT-Raman spectra of base-on MeCbl and Me-d_3-Cbl in aqueous solution (15 mM). Inset: High-frequency region of the spectrum showing the band for the CD_3 symmetric stretching mode of Me-d_3-Cbl.

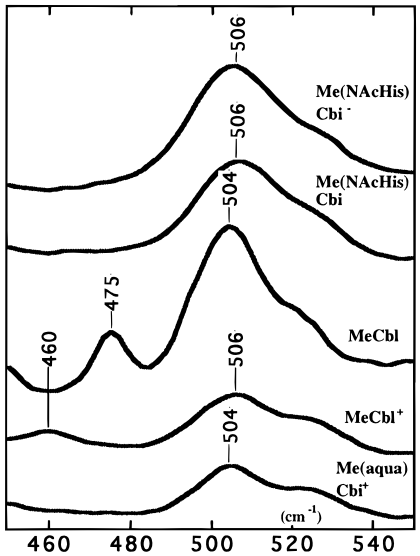

Figure 4. Dependence of the intensity of the Co−CH_3 stretching band of methylcobalt(III) corrinoids on the axial ligand, H_2O, *N*-AcHis$^-$, or DMBz. Peak heights are relative to the ~1495 cm^{-1} band (not shown). The band at ~475 cm^{-1} was not assigned; it was observed most prominently in spectra of MeCbl samples and is not shifted in Me-d_3-Cbl spectra. A ~525 cm^{-1} band or shoulder was observed in all the corrinoid spectra. Conditions: [MeCbl], 15 mM; [Me(*N*-AcHis)Cbi], 60 mM (with total [*N*-AcHis] 1.97 M at pH 10); [$MeCbl^+$], 100 mM; [$MeCbi^+$], 57 mM.

the red shift to imidazole deprotonation. We assign these 527 nm peaks to the β peak[20] (the band arising from the first vibrational overtone of the lowest energy transition) since it is similar to the β peak at 522 nm in the spectrum of MeCbl. This similarity supports the concept that the electron donation by neutral imidazole species is similar to the donation by DMBz.

Coordination and deprotonation of *N*-AcHis$^-$ is relevant to the imidazolate-like species that may be involved in the catalytic cycle of methionine synthase.[4] The Raman data (Table 1) suggest no significant weakening of the Co−CH_3 bond in the imidazolate form. The absence of a frequency change suggests that the biological role for the substitution of DMBz by imidazole is not related to an alteration of the ground state properties of the methylcobalt(III) species. It has been suggested that an imidazolate-like species coordinated to the Co of the bound cofactor may increase the nucleophilicity of the bound Co(I) form, facilitating methylation of the Cbl without destabilizing the methylcobalt(III) form.[4] In the absence of enzyme, the cofactor is not methylated by the natural biological reagent methyltetrahydrofolate. There is another possible role for imidazole. Reprotonation of the imidazole *via* the "catalytic quartet" may pull the ligand away from the cobalt, thus stabilizing the Co(I) form and facilitating methyl transfer from the Co(III) cofactor.[4]

Although intensity changes are difficult to interpret in a fundamental way, it is interesting that intensity changes were also observed in the cobaloxime[9,10] and bpb[11] B_{12}-model complexes. The intensity does seem to be a useful qualitative means for characterizing the nature of the *trans* ligand. This *trans* ligand effect on intensity does not seem to be eliminated by the difference [corrin *vs* $(DH)_2$] in the axial Co−N bond lengths (~0.1 Å) or the rather large difference in the equatorial ligands. However, the dependence of the band intensity and the similar frequencies of the Co−CH_3 stretching mode do reflect the finding that the Co−C bond lengths in models and B_{12} derivatives are similar.[12,21]

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Assignment of Corrin Modes. There are four bands of interest in the region of corrin ring modes. The band at ∼**1495 cm**$^{-1}$ is the most intense B_{12} Raman band. This band and the one at ∼**1545 cm**$^{-1}$ have been assigned in a resonance Raman[22] study as in-phase stretchings of the double bonds along the longer C5−Co−C15 axis and the shorter C10-Co axis of the corrin ring,[22] respectively. These assignments were based on selective enhancement of one band with visible light and the other by UV light. The two different $\pi-\pi^*$ corrin transitions associated with each electronic absorption band are known to be polarized along one of these axes. Also, the decrease in in-phase double-bond stretching modes in polyenes with increasing chain length supported the assignments to the corrin long and short modes.[22] Acid-catalyzed H/D exchange at C10H is known.[23] The NMR signal of C10H slowly disappeared and the bands shifted in several samples only if the sample was kept under acid/D_2O conditions or had previously been subjected to such conditions (see Table 1). This band shift demonstrates that the exchange is indeed acid-catalyzed and that the previous assignment of these bands to corrin ring modes is reasonable.

There is no mention in earlier Raman studies of the assignment of the band at ∼**1570 cm1**. In the absence of normal-mode coordinate analysis and isotopic labeling, the specific assignment of this band remains in question. In studies of compounds with structures similar to those of cobalamins,[24,25] bands in this region have been attributed to corrin skeletal modes. A direct comparison of a MoO corrin and a MoO porphyrin[26] also led to the assignment of bands in this region to C═C modes. An infrared band very similar in frequency at 1568 cm^{-1} has also been assigned as a corrin ring mode.[27] Our observation of the dependence of the frequency of this band on exchange of C10H to C10D (see above) confirms our assignment of this Raman band to a corrin ring mode. The band shifts to 1565 cm^{-1} in acidic D_2O for MeCbl and MeCbi$^+$, while for MeCbi$^+$ in D_2O at pH 6.5, the band at 1575 cm^{-1} remains close to the frequency in H_2O (Table 1 and Figure 5).

We believe the band at **1600 cm**$^{-1}$ that shifts to 1603 cm^{-1} upon acidification or removal of the nucleotide loop is possibly also a corrin ring band. This is the region for C═C modes, as mentioned above.[28] It is unlikely that the band is a DMBz mode since it is also seen in MeCbi$^+$.

***Trans* Ligand Dependence of the Band at ∼1495 cm^{-1}.** The corrin long-axis in-phase stretching band at ∼1495 cm^{-1} of methylcobalt(III) corrinoids did not change upon variation of the *trans* axial ligand (Table 1). This insensitivity suggests that there is no bending of the long axis.

***Trans* Ligand Dependence of the Band at ∼1545 cm^{-1}.** The frequency of the corrin short-axis in-phase stretching band, observed at 1544 cm^{-1} for base-on MeCbl (Figure 3), varied with the *trans* axial ligand. For the base-off form and for MeCbi$^+$, it shifted to 1538 cm^{-1} (Figure 6). Upon coordination of *N*-AcHis$^-$ to MeCbi$^+$, the band shifted (to 1548 cm^{-1} at pH 10 (Figure 6) and 1549 cm^{-1} at pH 14).

The frequency of the band at ∼1545 cm^{-1} increased in the order

$$\bar{\nu}_{\text{aqua}} < \bar{\nu}_{\text{DMBz}} < \bar{\nu}_{N-\text{AcHis}^-} \sim \bar{\nu}_{N-\text{AcHis}^{2-}}$$

Since the frequency changes did not follow a trend correlating well with ligand size, these shifts may also be attributed to electron donor ability. The slight difference in frequency of the band at ∼1545 cm^{-1} between base-on MeCbl and Me(*N*-AcHis)Cbi (Figure 6), which have ligands with similar electron-donor abilities, may be indicative of a secondary structural effect. This possibility is discussed below.

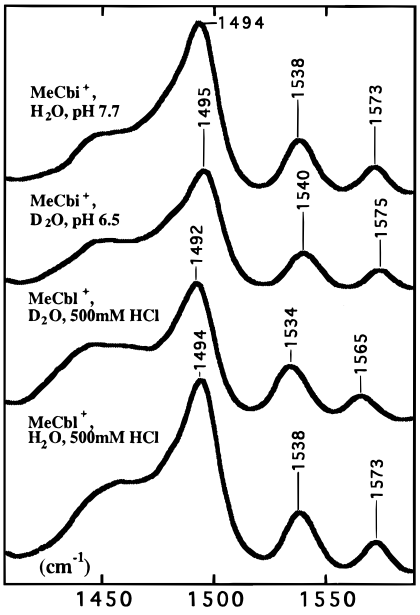

Figure 5. Effects of exchange in D_2O or acidic D_2O on the frequencies of the corrin-ring modes in the FT-Raman spectra of methylcobalt(III) corrinoids, 60−100 mM.

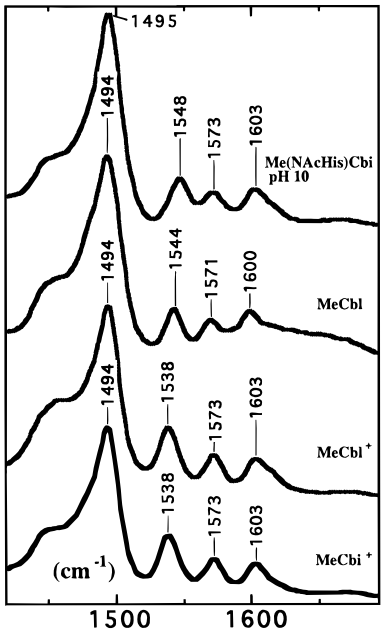

Figure 6. Effects of the axial ligand on the frequencies of the corrin-ring modes in the FT-Raman spectra of methylcobalt(III) corrinoids in H_2O.

***Trans* Ligand Dependence of the Band at ∼1570 cm^{-1}.** The band at ∼1570 cm^{-1}, probably a corrin ring mode (see above), was found to be insensitive to the nature of the neutral axial ligand.

***Trans* Ligand Dependence of the Band at ∼1600 cm^{-1}.** Deligation of the bulky DMBz by acidification of MeCbl or by loop cleavage increased the frequency of the band at ∼1600

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cm^{-1} to 1603−1604 cm^{-1}; this frequency then changed little upon coordination of histidine (Table 1). Even in 1 M NaOH (Table 1), the frequency remains at the base-off/$MeCbi^+$ value! Indeed, the frequency of this corrin band lies within a relatively small range for all base-on cobalamins in solid (1597−1602 cm^{-1}) and in solution (1599−1600 cm^{-1}) states, except that for cyanocobalamin (unpublished results). The frequency of the band may be indicative of the base-on/base-off conformation of the corrin, and the possibility that this band is a core-size marker is discussed next.

Core Size and Butterfly Bending Effects. Raman spectroscopy has proved to be very informative about core size effects in another important class of biomacrocyclic complexes, metalloporphryins.[29] Core-size changes in porphyrins have been attributed in part[29] to a "doming" effect, in which the porphyrin undergoes an umbrella-like distortion. The core size is measured by the porphyrin center-to-pyrrole nitrogen distance, C_t-N. Good correlations between core size and frequencies of various bands in different porphyrins containing different metals and axial ligands have been found.[30] Three bands in the 1500−1615 cm^{-1} region of porphyrins have been assigned to in-plane skeletal modes as follows:[29] $\nu_{C_b-C_b}$ (A_{1g}), $\nu_{C_b-C_b}$ (B_{1g}), and $\nu_{C_a-C_m}$ (A_{2g}), where C_a and C_b are the carbons in the α and β positions, respectively, from the pyrrole nitrogens, and C_m is the methine carbon. In each of these cases an inverse correlation has been found between the Raman frequency and the crystallographically determined core size of the porphyrin;[30] i.e., the core size increases with decreasing Raman frequency. The magnitude of this effect in porphyrins is typically ∼0.01 Å core-size change/∼3 cm^{-1} frequency change.[30] If the 1600 cm^{-1} corrin band follows similar trends, the core size of the corrin decreases when DMBz dissociates. A plot of the frequencies of these bands versus the core size of the absorbing porphyrins has been shown to follow the relationship $\bar{\nu} = K(A - d)$, where $\bar{\nu}$ is the frequency, K is the slope, d is the core size, and A is the intercept.[30] The slope does not vary greatly with the band or the porphyrin.

Although there is a lack of X-ray structures of cobalt corrinoids sufficiently accurate to determine whether any relationships exist between core size and Raman frequency, the core size does not seem to change much. In X-ray structures, differences in the Co−N23 bond length exist between the normal base-on cobalamins[12,31−33] and CN(Im)Cba [Co−β-cyanoimidazolylcobamide, in which the nucleotide loop has an imidazole in place of the DMBz].[32] The short Co−N (ring C) bond length for the Cba [1.853(9) Å] contrasts with those of cyano-Cbl[32] at 1.917(9) Å, MeCbl[12] at 1.93(2) Å, coenzyme B_{12}[33] at 1.910(14) Å, aqua-Cbl perchlorate[31] at 1.904(2) Å, and (adeninylpropyl)-Cbl[34] at 1.886(7) Å. However, these differences are not statistically significant so the X-ray studies do not clearly indicate whether the core size changes.

The bands at ∼1570 and ∼1545 cm^{-1} are sensitive to H/D exchange (see above). Therefore the band at ∼1570 cm^{-1} may also be a $\nu_{C_a-C_m}$ type of mode, since C10 is a methine carbon. The frequency of the corrinoid band at ∼1570 cm^{-1} shifts only slightly, if at all, when the axial ligand changes, although there is a significant effect on the frequency of the short-axis band at ∼1545 cm^{-1}. The latter is quite sensitive to changes in axial ligand in the order aqua < DMBz < *N*-AcHis (see Table 1), while the long-axis band at 1495 cm^{-1} is not affected. The relationship follows mainly that expected for electron donation, not axial ligand bulk; therefore there is no significant effect of the axial ligands on the core size in these cobalt corrinoids.[30]

In contrast to the umbrella-like distortion of porphyrins, the corrins, which are less symmetrical, are buckled with a propensity to undergo a butterfly-like folding,[35] the short axis being the crease in the fold with upward bending toward the β side. The small increase in the frequency of the 1545 cm^{-1} band from MeCbl to Me(*N*-AcHis)Cbi suggests better π bonding within the corrin for $MeCbi^+$ and may be due to a flattening corrin. Since the long-axis-mode frequency remains unchanged, the short-axis-mode frequency increase may be due to a perturbation along the Co−C10 axis (see Figure 1). The recent study[32] comparing vitamin B_{12} with CN(Im)Cba demonstrated some significant structural changes. Spectroscopic and crystal structure data were compared to those of vitamin B_{12} to assess the effects of the DMBz on the corrin ring. Generally speaking, replacement of DMBz with imidazole in the loop results in the corrin ring assuming a more planar conformation. The cobalt ion is located in the plane of the equatorial nitrogens; all of the C_α positions except one are closer to the plane; and the "upward folding" of the corrin is reduced from 18° to 11 when imidazole is coordinated. The Raman data in our study suggest that changes in the methylcobalt(III) corrinoids may be similar to those in the cyanocobalt(III) corrinoids.

Amide I Bands at ∼1670 cm^{-1}. We assign the band at ∼1670 cm^{-1} (Table 1) in all the spectra of the cobalt corrinoids in H_2O to the overlapping amide I C═O stretching mode of the propionamide and acetamide side chains since these should have similar frequencies and intensities.[25]

In an FT-IR study,[27] a band found between 1675 and 1679 cm^{-1} for MeCbl, cyano-Cbl, hydroxo-Cbl, and $(CN)_2$Cbi in ethanol was assigned as the amide I C═O stretching mode of the propionamide and acetamide side chains. In D_2O, the FT-IR band for all of the cobalt corrinoids was found to lie within the range 1630−1633 cm^{-1}. This 43 cm^{-1} shift to lower frequency from ethanol to D_2O for MeCbl was attributed to a greater contribution of the resonance form in which the carbonyl oxygen carries a negative charge while the amide nitrogen has a positive charge in the more polar, better H-bonding solvent, D_2O.[27]

In this work, we observed a Raman band for MeCbl at 1639 cm^{-1} in D_2O and at 1680 cm^{-1} in ethanol (Table 1). This Raman shift is close to the IR shift cited above. However, we observed the band for base-on MeCbl at 1670 cm^{-1} in H_2O. If the shift were due primarily to changes in the distribution of resonance forms as implied in the IR study, then the frequencies would be similar in H_2O and D_2O. The 31 cm^{-1} shift we found for H_2O vs D_2O is typical for the $-NH_2$ to $-ND_2$ isotopic substitution in acetamide.[36] Therefore, we attribute most of the shift in ethanol vs D_2O to H/D exchange to form $-COND_2$. Other factors, including the increased contribution of the charge-separated resonance form, have a smaller influence on the shift.

DMBz Band at ∼1315 cm^{-1}. A band at 1312 cm^{-1} for MeCbl completely vanishes for the base-off form (Figure 7). The band is found at 1310 cm^{-1} in the solution spectrum of $(CN)_2Cbl^-$ (Figure 7) and (as a shoulder) in this region in the spectrum of aquacobalamin. This band was also observed in

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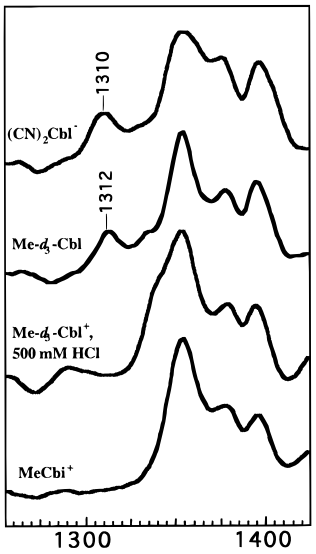

Figure 7. Regions of the FT-Raman spectra containing the 1315 cm^{-1} band. $(CN)_2Cbl^-$ (81 mM) was formed by addition of 374 mM KCN, and spectral data from a sample of 293 mM KCN were subtracted.

spectra of solid adenosyl- and cyanocobalamin[8] and in resonance-enhanced spectra of (photolyzed) MeCbl.[22] Its complete absence in the spectra of base-off $MeCbl^+$, of $MeCbi^+$ with and without coordinated *N*-$AcHis^-$, and of $(CN)_2Cbi$ indicates that this band is a mode involving the nucleotide loop. The presence of the band in the $(CN)_2Cbl^-$ spectrum, in which the DMBz is base-off but deprotonated, suggests that this is a benzimidazole ring stretching mode; the band for such a mode has been found in this region.[37] The band is apparently sensitive to protonation but not to coordination.

Methyl-d_3 Symmetric Stretch at ~2105 cm^{-1}. The isotopically sensitive CH_3 symmetric stretching band, which shifted from 2897 to 2106 cm^{-1} for $pyCo(DH)_2CH_3$ compared to $pyCo(DH)_2CD_3$,[9] was seen clearly at 2110 cm^{-1} for Me-d_3-Cbi^+, at 2109 cm^{-1} for base-on Me-d_3-Cbl (Figure 3), and at 2103 cm^{-1} for Me-d_3-(*N*-AcHis)Cbi^-, pH 13. This Me-d_3 band may be a useful diagnostic tool in studies of enzyme-bound Me-d_3-Cbl since that region of the spectrum is devoid of other bands. Unfortunately, the other isotopically shifted band associated with the Co−methyl C−H bonds, the CH_3 bending mode seen at 1176 cm^{-1} for model Co−CH_3 compounds and at 892 cm^{-1} for the Me-d_3-Cbl models,[9] could not be detected in the cobalt corrinoids studied here.

Summary of Spectral Results. The frequency of the Co−C stretching mode at ~505 cm^{-1} (Table 1) was only minimally affected by the *trans* ligands (L). However, the intensity of this band, relative to that of the intense band at ~1495 cm^{-1}, doubled when the axial water was replaced by an N-donor ligand.

Our C10H to C10D isotopic exchange studies confirmed that three bands in the region 1480−1580 cm^{-1} are due to corrin modes. Bands at ~1495 cm^{-1} and at ~1570 cm^{-1}, corrin ring modes, were insensitive to L. The frequency of the corrin short-axis in-phase mode at ~1545 cm^{-1} was sensitive to L. This band could be influenced to a small extent by structural changes in the corrin. A fourth band, probably also due to a corrin mode, was found near this region; it is at 1600 cm^{-1} for base-on MeCbl (Table 1) but at 1603−4 cm^{-1} for all other methylcobalt(III) corrinoid species. These either were base-off or had imidazole coordinated. This band may also have some sensitivity to the steric effects of L. However, the lack of a consistent relationship of shifts to steric bulk of L for the four bands suggests that electronic properties of L dominate the corrin ring spectral changes and that structural changes in the corrin are small.

A band at ~1315 cm^{-1} found only in the spectra of cobalamins in which the DMBz has not been protonated is a DMBz band. The CD_3 symmetric stretching frequency of the Co−CD_3 group lies in a region (~2105 cm^{-1}) of the spectrum that is usually devoid of other bands commonly found in proteins. The frequency of this band is sensitive to the electronic nature of the axial ligand.

The frequency of the amide I band of MeCbl in H_2O (measurable by FT-Raman but not by FT-IR spectroscopy) is only ~10 cm^{-1} from that in ethanol. Thus, H/D exchange to form −$COND_2$ causes ~30 cm^{-1} of the ~40 cm^{-1} shift in the amide I FT Raman band between ethanol and D_2O. Likewise, the reported ~43 cm^{-1} shift in the amide I IR band between ethanol and D_2O results primarily from H/D exchange. We attribute only ~10 cm^{-1} of this shift to other factors, including H-bonding and the increased contribution of the charge-separated resonance form.

Conclusions

Our main goal was to determine the effects of the *trans* ligand, especially the effect of replacing DMBz with imidazole or imidazolate, on the properties of the cofactor so that the biological role of DMBz substitution by imidazole in enzyme functions can be understood. As a first step, we developed an efficient approach to prepare derivatives. Preparation of $MeCbi^+$ from MeCbl has been found to be more convenient by the trifluoromethanesulfonic acid method than by the $Ce(OH)_3$-catalyzed method. Imidazolate MeCbi complexes could be formed under strongly basic conditions. An ~10 nm red shift in the 527 nm β peak in the UV/vis spectra of imidazole and *N*-AcHis MeCbi complexes was observed when the solution basicity was increased to 1 M NaOH, but the *N*-MeImd complex did not show the shift; these results provide strong evidence for the formation of imidazolate complexes. Before this study, there was essentially no evidence for imidazolate bound to organocobalt corrinoids.

The replacement of DMBz by imidazole and imidazolate has only small effects on Raman bands of both the axial bond and the corrin. These spectral results suggest that the reason that imidazole is substituted for DMBz does not involve the methylcobalt(III) corrinoid ground state species. Instead, the properties of imidazole, such as its ability to form a strong imidazolate-like donor or its small steric size, could be important for an intermediate or an activated complex in the catalytic cycle.

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