Resonance Raman Co−C Stretching Frequencies Reflect Bond Strength Changes in Alkyl Cobalamins, but Are Unaffected by *Trans* Ligand Substitution

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We report resonance Raman (RR) spectra of methyl-, ethyl-, and adenosylcobalamin (MeCbl, EtCbl, and AdoCbl, Figure 1), in which the Co−C stretching vibration is identified by isotopic substitution. The frequency of this mode diminishes in the order MeCbl > EtCbl > AdoCbl, consistent with changes in the Co−C bond dissociation energy (BDE). Unexpectedly, however, the frequency is unaffected by displacement of the benzimidazole *trans* ligand. These spectra provide important controls for the investigation of mechanism in vitamin B_{12}-dependent enzymes.[1]

Although the corrin chromophore provides strong enhancement of RR scattering, early B_{12} studies were hampered by Co−C bond photolysis induced by the Raman laser.[2] The later discoveries that the Co−CH_3 stretch can be detected in B_{12} models[3] and is a prominent feature of the MeCbl FT-Raman spectrum obtained with long-wavelength (1064 nm YAG laser) nonphotolyzing radiation[4] led to investigations of the determinants of the Co−CH_3 bond strength.[5] However, other Co−alkyl stretches have escaped detection with FT-Raman spectroscopy.[4] Moreover, the lack of resonance enhancement means that high sample concentrations are required for spectral acquisition, precluding studies on B_{12}-containing proteins.

Recently, we were able to detect the Co−CH_3 stretching RR band in a B_{12}-dependent methyl transferase enzyme, using a cryogenic technique.[6] We now extend this technique to alkyl cobalamins (Figure 1). Although the RR spectra contain numerous bands arising from the corrin chromophore, isotopic substitution permits ready detection of the Co−C stretches at 506, 471, and 443/429 cm^{-1} for Me-, Et-, and AdoCbl, respectively (Figure 2). The first of these values agrees with the FT-Raman data,[4] but the other frequencies have not previously been determined. The pair of isotope-sensitive bands seen for AdoCbl most likely results from two equilibrating conformers, having different adenosyl orientations, which have been detected by NMR spectroscopy.[7]

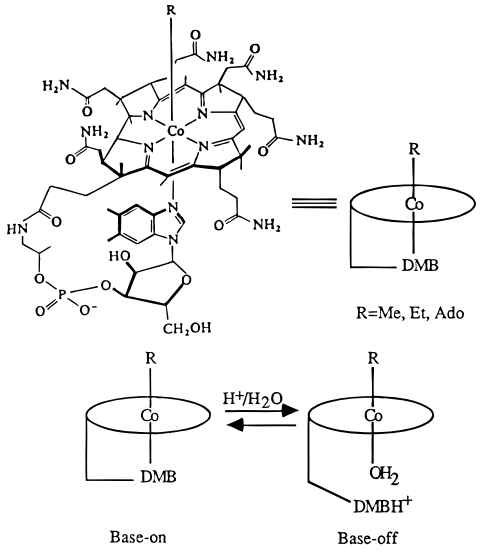

Figure 1. Structural diagram of alkylCbl species and a schematic representation of the base-on/base-off equilibrium. In the base-off form, H_2O binds to the Co at low temperature but not at room temperature.[20]

The trend in the Co−C frequencies (MeCbl > EtCbl > AdoCbl) implies weakening of the Co−alkyl bond with increasing bulk of the alkyl group, consistent with prevalent ideas about steric effects.[8] The AdoCbl[9] and MeCbl[10] crystal structures show the corrin ring to be folded, butterfly fashion, with the wings tilted toward the alkyl group, enforcing nonbonded contacts. The Co−alkyl bond dissociation energy is reported to be appreciably lower for AdoCbl (30 ± 2 kcal/mol)[11] than for MeCbl (37 ± 3 kcal/mol).[12] If this lowering is attributed to Co−C bond weakening, due to increased nonbonded forces on the bulky adenosyl group, then a proportionate lowering of the Co−C force constant is expected. Consistent with this expectation, the ratio of the BDE values (AdoCbl:MeCbl = 0.81 ± 0.1) is the same as the estimated force constant ratio[13] (0.83, taking the mean of the two observed frequencies 443 and 429 cm^{-1} as representative of AdoCbl). Thus, the RR spectra indicate that the BDE lowering is mainly due to Co−C bond weakening in AdoCbl, and, by extension, in EtCbl as well.

However, the RR spectra also reveal that effects of the *trans* ligand on the Co−alkyl BDE are *not* connected to the Co−C bond strength (Figure 3). When the benzimidazole ligand is

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(13) We calculate Co−C force constants *F* of 1.85, 1.77, and 1.50 mdyn/Å for MeCbl, EtCbl, and AdoCbl from the diatomic oscillator approximation $\nu(cm^{-1}) = 1302.83\ [F(\text{mdyn/Å}/\mu)]^{1/2}$, where $1/\mu = 1/M_{CO} + 1/M_L$. M_L is the effective mass of the alkyl ligand calculated from the observed isotope shifts. For MeCbl, the 506 cm^{-1} band shifted to 493 cm^{-1} upon $^{13}CH_3$ substitution (not shown), giving $M_L = 15.4$ for the $^{12}CH_3$ group. [A higher value of 18.3 is obtained from the 506 → 478 cm^{-1} shift upon CD_3 substitution, probably reflecting some mixing with CD_3 coordinates, as evidenced by CD_3 shifts in bands at 1156 and 789 cm^{-1} (not shown). Isotope sensitivity of additional bands was not observed in the other Cbl spectra.] For AdoCbl, $M_L = 17.3$ was calculated from the average νCo−C (436 → 418 cm^{-1}) upon deuteration of the Co-bound CH_2 group (increase in M_L by two mass units). For EtCbl, $M_L = 17.5$ from the 471 → 452 cm^{-1} shift upon CD_2CH_3 substitution. For MeCbl and AdoCbl, the estimated force constant is proportional to the reported BDE values, and if this relation also holds for EtCbl, then the predicted BDE for EtCbl is 35 kcal/mol.

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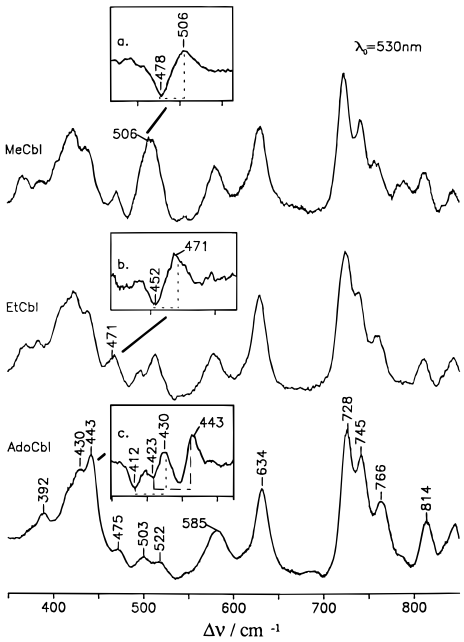

Figure 2. Cryogenic, resonance Raman spectra of base-on CH_3-, CH_2CH_3-, and AdoCbl in 50mM pH 7.5 potassium phosphate buffer. Isotope labeling identifies the Co−C stretching band, as shown in the inset difference spectra: (a) CH_3Cbl/CD_3Cbl; (b) CH_3CH_2Cbl/CH_3CD_2Cbl; (c) AdoCbl/5′CD_2-AdoCbl. CH_3Cbl and AdoCbl were purchased from Sigma, while EtCbl was synthesized as described for neopentylcobalamin.[21] CH_3CH_2Br, CD_3I, and $^{13}CH_3I$ were purchased from Aldrich, and CH_3CD_2Br was from Cambridge Isotopes Lab. AdoCbl with deuterium at the 5′ position was synthesized enzymatically using ribonucleotide reductase.[22] The cobalamins were purified by HPLC on a C18 reverse phase column (Phenomenex) eluted (2 mL/min) isocratically with 5 mM HCl, pH 5.3 (adjusted with NaOH), 40% methanol. The cobalamin fractions (detected by absorption at 254 nm) were pooled, lyophilized, and stored at −80 °C, and then dissolved in cold degassed 50 mM potassium buffer, pH 7.5, just before resonance Raman experiments. Samples (20 μL, 1−3 mM) were loaded in a liquid N_2 dewar[23] under N_2 gas in the dark, and then frozen and degassed by pumping. Raman scattering from a Kr^+ ion laser (530.9 nm) was collected in a 135° backscattering geometry, dispersed in a triple monochromator (3 cm^{-1} spectral width), and detected with an intensified photodiode array. Raman shifts were calibrated with CCl_4 and *N*,*N*-dimethylformamide standard spectra. Sample integrity was checked via UV-visible absorption and HPLC analysis after the Raman measurement.

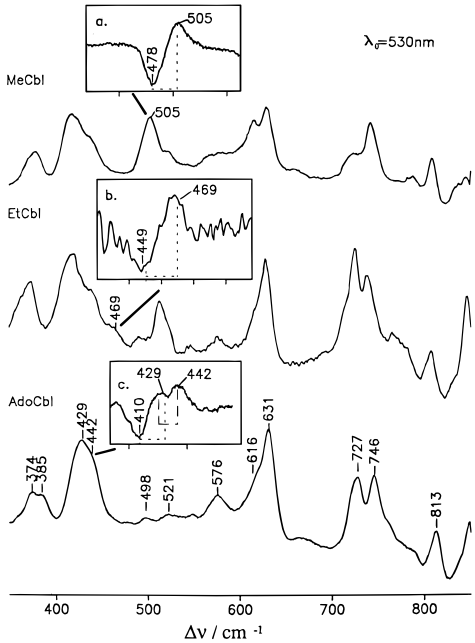

Figure 3. RR spectra of base-off CH_3-, CH_2CH_3-, and AdoCbl in 0.1 N HCl, with inset difference spectra: (a) CH_3Cbl/CD_3Cbl; b. CH_3CH_2Cbl/CH_3CD_2Cbl; c. AdoCbl/5′CD_2-AdoCbl. Experimental conditions were as in Figure 2. The weakness of the 469 cm^{-1} band in the EtCbl spectrum reflects partial photolysis in this sample (∼30%, as judged by HPLC), which was the least stable sample in the series.

replaced by water, upon protonation in acid solution ["base-off" cobalamin, see Figure 1] the Co−Ado BDE is increased substantially to 34.5 ± 1.8 kcal/mol.[14] But there is *no* change in the frequency of the Co−Ado stretching bands in the base-off form, although their relative intensities are altered, and there are other changes in the RR spectrum, probably due to corrin conformation changes. Likewise, the Co−Et and Co−Me band positions are unaffected by benzimidazole displacement. This result for MeCbl has also been obtained via FT-Raman by Marzilli and co-workers,[15] who found, in addition, that the Co−Me frequency was unaffected by coordination of imidazole or imidazolate. Thus the *trans* ligand does *not* control the Co−alkyl bond strength, as has often been assumed. The increased BDE of base-off AdoCbl must instead reflect destabilization of the products of the bond dissociation reaction, namely cobalt(II)corrin and adenosyl radical.

These results have important implications for the issue of Co−alkyl bond activation in B_{12} enzymes.[1,8] The role of the *trans* ligand is highlighted by EPR spectra of methylmalonyl-CoA mutase,[16] showing the benzimidazole to be displaced from AdoCbl by a nitrogenous ligand, and by the crystal structures of methylmalonyl-CoA mutase[17] and of methionine synthase,[18] which show the benzimidazole of Cbl to be displaced by a histidine side chain. The present evidence indicates that these displacements need not have any influence on the Co−C bond strength, although they could nevertheless affect the enzymatic rate. For example, a charge relay mechanism has been suggested for methionine synthase in which proton transfer between the histidine ligand and an adjacent aspartate side chain could alternatively stabilize the Co(III) ground state or the Co(I) intermediate produced by methyl transfer.[19] On the other hand, the one Co−CH_3 frequency so far detected in a B_{12} enzyme, the corrinoid-FeS protein in the acetyl-CoA synthesis pathway,[6] is much lower (429 cm^{-1}) than in enzyme-free MeCbl and implies substantial enzyme-induced Co−CH_3 bond weakening (by ∼7 kcal/mol if BDE scaling of the force constant applies). Some mechanism other than (or in addition to) benzimidazole displacement is implied, possibly an influence on the corrin ring conformation that weakens the Co−CH_3 bond sterically.

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