Co−C Bond Activation in B_{12}-Dependent Enzymes: Cryogenic Resonance Raman Studies of Methylmalonyl-Coenzyme A Mutase

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Abstract: The determination of its crystal structure, as well as extensive biochemical characterizaion, has focused attention on methylmalonyl-coenzymeA (MM-CoA) mutase as a paradigm for AdoCbl (adenosylcobalamin)-dependent enzymes. These enzymes catalyze carbon skeleton rearrangement reactions via a radical mechanism, which originates in homolysis of the Co−C bond. The determinants of this mechanism are of great chemical and biochemical interest. We report resonance Raman (RR) spectra of MM-CoA, in the presence of substrates and inhibitors, using a cryogenic technique to prevent laser-induced photolysis of the Co−C bond. RR spectroscopy provides an in-site probe of cobalamin structure. Although the spectra are dominated by the corrin ring, four RR bands arising from Co-bound adenosine can be detected via isotope editing. These are assigned to the Co−C bond stretch, the Co−C−C angle bend, a 5′-C-coupled ribose deformation, and a hindered rotation of the adenosine about the Co−C bond. The RR spectra confirm enzyme-catalyzed H exchange between substrate, but not inhibitors, and the adenosyl 5′-C atom. The RR enhancement pattern is affected in different ways by binding inhibitors or slow substrates on the one hand and product or substrate on the other. Since product dissociation is apparently rate-limiting and the rearrangement equilibrium lies toward product, the adducts with product or substrate represent the product state (P state), whereas those with inhibitors or the slow substrates represent the substrate state (S state). The RR enhancement changes (activation of δCoCC and weakening and downshift of νCoC) indicate Co-Ado tilting, to a small extent in the resting enzyme and to a larger extent in the S state. These changes are reversed in the P state. Thus Co-Ado tilting is identified as a contributor to activation of the Co−C bond in the S state. The steric forces that induce tilting are apparently relaxed in the P state, thus promoting the rearrangement of substrate to product. The corrin vibrational modes are responsive to the ring conformation but are mostly unaffected by substrate or inhibitor binding, indicating that Co−C activation does not involve corrin conformation changes. However, two modes, whose frequencies (423 and 437 cm^{-1}) are consistent with contributions from Co−N(corrin) stretching, shift down in the P state, suggesting displacement of the Co from the corrin plane. This effect may result from lengthening of the Co−N(histidine) bond, as seen in the crystal structure.

Introduction

Coenzyme B_{12} has long fascinated chemists because of its complex structure (Figure 1) and because it offers the only biological instance of a stable organometallic bond. Moreover there are two different B_{12} coenzymes, methylcobalamin (MeCbl) and 5′-deoxyadenosylcobablamin (AdoCbl), whose mechanisms are fundamentally different.[1] Enzymes utilizing MeCbl catalyze the transfer of a methyl cation equivalent, via heterolysis of the Co−C bond,[2] while those employing AdoCbl catalyze radical-induced rearrangement reactions (or ribonucleotide reduction[3]), via homolysis of the Co−C bond.[4] How the Co−C bond is activated toward homolysis or heterolysis is an enduring subject of research. The degree of activation is spectacular, since the Co−C bond is ordinarily quite stable. Thus the Co−C dissociation enthalpy is 31.5 ± 2 kcal/mol for AdoCbl

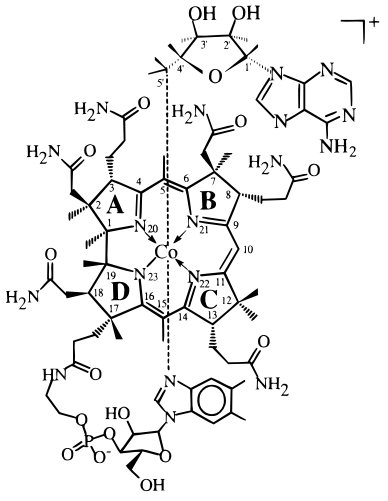

Figure 1. Structure of Coenzyme B_{12}.

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and the thermal homolysis rate at 25 °C is only 4×10^{-10} s^{-1}.[5] In AdoCbl-dependent enzymes this rate is increased by factors of $10^{12\pm1}$, implying a ~15 kcal/mol destabilization of the Co−C bond.[5−8]

The enzyme can accomplish this activation by destabilizing the AdoCbl ground state or by stabilizing the transition state for homolysis, or both. Two classes of activation mechanism have been explored, steric and electronic. Steric mechanisms weaken the Co−C bond via nonbonded forces, and are perforce ground-state destabilizing mechanisms. Two effects have been considered, steric repulsion of the bulky adenosyl ligand by the corrin ring,[9−14] whose conformation can be altered in the protein binding pocket, and tilting of the Co−C bond[15] as a result of steric forces on the adenosine from protein residues or from bound substrate. Electronic mechanisms focus instead on the relative stabilization of AdoCbl and of the homolysis product, cob(II)alamin (Co[II]Cbl), by the axial base.[16] In the free cofactor this base is dimethylbenzimidazole (DMB), which is tied to the corrin ring by a covalent linkage (Figure 1), but recent crystal structures,[17,18] as well as EPR measurements,[19] have revealed that the DMB can be replaced by a histidine side chain in B_{12}-dependent enzymes. Studies of the free cofactor establish that variations in the axial base do influence the Co−C homolysis rate,[7−9] but they do not alter the Co−C force constant.[20−22] Thus the axial base effect involves transition-state stabilization, at least outside the enzyme.

Resonance Raman (RR) spectroscopy can illuminate these issues by reporting the vibrational frequencies and intensities of the coenzyme in situ. Vibrational modes of the corrin ring are sensitive to the corrin conformation, while modes of the Co-bound adenosyl report on the ligand geometry; the Co−C stretching frequency is a direct reporter of the bond strength in the ground state. Although the RR spectra are dominated by corrin modes,[23−25] enhanced via resonance with the corrin π-π^* transitions,[26] Co-Ado modes are also coupled to these transitions, albeit weakly, and can be isolated from corrin contributions by isotope editing.[20,21] A technical obstacle to RR studies, laser-induced photolysis, can be overcome with cryogenic techniques.[20]

Scheme 1. Isomerization of MM-CoA Catalyzed by MM-CoA Mutase

In this study, we focus on methylmalonyl coenzyme A (MM-CoA) mutase, a prototype of AdoCbl-dependent isomerase enzymes.[1] It is the only such enzyme found in both bacteria and mammals and catalyzes the difficult 1, 2-carbon skeleton rearrangement of MM-CoA to succinyl coenzyme A (S−CoA) (Scheme 1). Deficiency of MM-CoA mutase activity in the human body results in methylmalonic acidemia due to accumulation of methylmalonic acid.[27] The bacterial enzyme has been studied extensively,[1] and crystal structures are available.[17] We have recently reported cryogenic RR spectral changes when AdoCbl is bound to the mutase; the corrin ring conformation is altered, and the Co−C bond is weakened, but only slightly.[28] We now report further spectral changes associated with the biochemically relevant substrate- and inhibitor-bound states of the enzyme and discuss their implications for the Co−C activation mechanism.

Experimental Section

Sample Preparation. Bacterial MM-CoA mutase was overexpressed in *E. coli* strain K38 and purified as previously described.[29] The recombinant expression vector (pMEX2/pGP1−2) harboring the *Propionibacterium shermanii* genes was a gift from Dr. P. Leadley (Cambridge University). Natural abundance AdoCbl, MM-CoA, S-CoA, and glutaryl-CoA (G-CoA) were purchased from Sigma. 5′-D_2-AdoCbl was synthesized by H/D exchange of AdoCbl in D_2O buffer catalyzed by ribonucleotide reductase (a gift from Prof. JoAnne Stubbe at MIT). 5′-^{13}C-AdoCbl was a gift from Dr. Kenneth L. Brown (Ohio University). Isotope-labeled enzyme was prepared by reconstitution with the corresponding isotope-labeled cofactor according to published procedures.[29] Ethylmalonyl-CoA (EtM-CoA) and cyclopropylcarbonyl-CoA carboxylate (CPC−CoA) (Chart 1) were synthesized as previously described.[30]

Protein samples for Raman studies were concentrated to 0.6−1.3 mM in coenzyme B_{12}. Sample solutions (20 μL) were loaded into sample cells in a liquid nitrogen dewar[31] under reduced light at 4 °C in a glovebag filled with nitrogen. The dewar was then pumped to prevent frost formation on the surface of the sample. The effects of binding substrate or substrate analogues were investigated by mixing enzyme solution with concentrated stock solution of the substrate or substrate analogue at 4 °C in the glovebag. The samples were cooled to liquid N_2 temperature within two minutes of mixing. The stock solutions were

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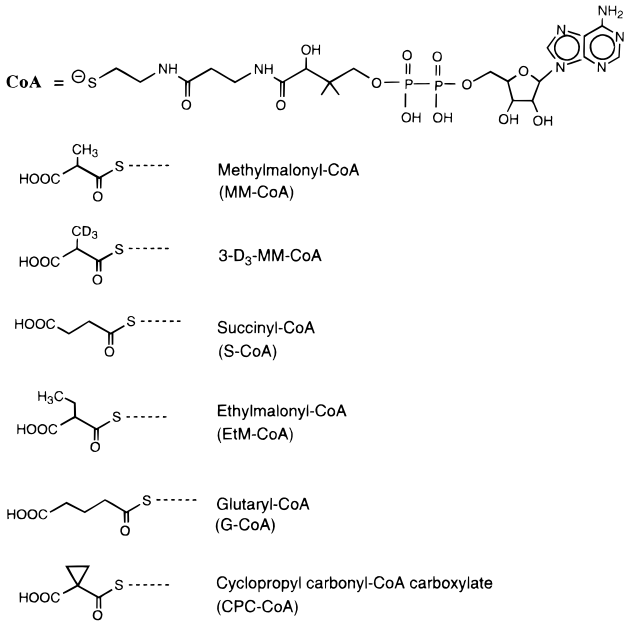
Chart 1

MMCoA Mutase Substrate and Substrate Analogues



prepared just before the Raman measurement by dissolving lyophilized substrate or substrate analogue in 1 M K_2HPO_4 with the final pH adjusted to about 7.5 with HCl or KOH. The concentrations of the stock solution were determined by the absorption at 260 nm (ϵ = 13.4 mM^{-1} cm^{-1}). To ensure that all enzyme molecules are bound to the substrate or substrate analogues, we added more than 30-fold excess of substrate or substrate analogue to the reaction mixture; control experiments established that the excess reagents did not contribute to the Raman spectra. Sample integrity was checked with UV−vis absorption spectroscopy after the Raman measurements.

RR Spectroscopy. Resonance Raman spectra were collected in ~135° backscattering geometry with a Spex 1877 triple monochromator equipped with an intensified photodiode array detector. Excitation was provided by a Kr^+ laser (Coherent Innova 100-K3A). The following conditions were used for all of the experiments: excitation wavelength = 568.2 nm, laser power at the sample = 20 mW, and spectral slit width = 3 cm^{-1}. Raman shifts were calibrated with known solvent spectra. Spectral analysis, including baseline correction, was carried out with Labcalc (Galactic) software. For precise comparison of different isotope-labeled proteins and proteins under different conditions, the samples to be compared were always loaded side by side on the liquid nitrogen cold-finger; their spectra were collected with the same instrumental settings. Difference spectra were obtained by normalizing the prominent and well-isolated 630 cm^{-1} corrin RR peak as reference.

Results

The cryogenic RR technique involves cooling the protein samples to liquid N_2 temperatures.[31] Substrate and substrate analogues (Chart 1) were mixed with holoprotein at 4 °C and frozen within two minutes. Reactions that are slow on this time scale were quenched, while faster reactions were not. Binding was assured by the high protein concentration (~1 mM) and by adding a 30-fold excess of the substrate or analogue. The K_d's are less than 100 μm (48.5 μM for EtM-CoA, 90 μM for CPC−CoA; Taoka, S.; Banerjee, R. Unpublished results). As with all cryogenic measurements, the possibility of freezing-induced structure changes must be kept in mind. Cofactor integrity was checked by recording absorption spectra of samples thawed after RR spectral acquisition.

1. Corrin Modes Are Unaffected by Substrate Binding. As reported previously,[28] the high-frequency modes of the corrin ring are altered in frequency and relative intensity when AdoCbl binds to MM-CoA mutase (Figure 2). The frequency upshifts of the bands above 1450 cm^{-1} resemble those seen in metalloporphyrins upon relaxation of out-of-plane macrocycle distortions,[32] and signal flattening of the corrin ring, as is observed in the MM-CoA mutase crystal structures.[17]

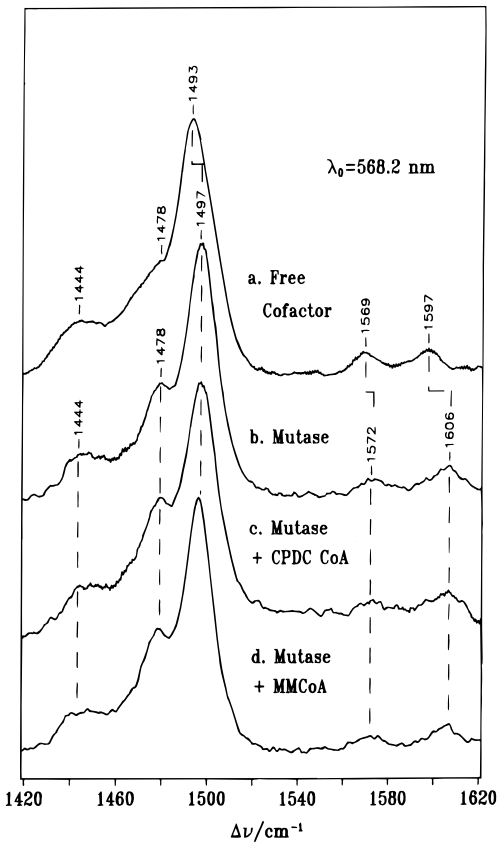


Figure 2. High-frequency corrin RR bands for AdoCbl free in solution (a), and bound to MMCoA mutase (b), in the presence of inhibitor (CPDCCoA) (c) and of product substrate (MMCoA) (d).

These bands remain at the higher positions when saturating concentrations of the substrate, MM-CoA, or of the inhibitor, CPC−CoA (Chart 1), are added to the holoenzyme (Figure 2). Thus there are no additional corrin conformation changes upon binding substrate or inhibitor.

2. Enhancement and Assignments of 5′-Deoxyadenosyl Ligand Modes. The low-frequency spectrum of MMCoA mutase (Figure 3) contains many corrin modes but is also sensitive to isotopic labeling of the Co-bound adenosyl group, establishing that some adenosyl modes are resonantly enhanced.[21] These modes can be isolated from the overlapped corrin modes by isotope editing. The difference spectrum for ^{13}C substitution clearly reveals two bands, which are readily assignable to the Co−C stretch (νCo−C), at 424 cm^{-1}, and to a deformation mode of the ribose ring (δribose), at 567 cm^{-1}, which involves motion of the 5′−C substituent. The latter frequency is the same as observed in the free cofactor, whereas the Co−C frequency is 6 cm^{-1} lower, indicating a slight weakening of the Co−C bond in the holoenzyme.[28]

Additional adenosyl modes are revealed by deuteration of the 5′-methylene group. This substitution shifts the δribose and νCo−C modes to a greater extent than 5′-^{13}C, reflecting contributions from C−H/D displacements. It also reveals two

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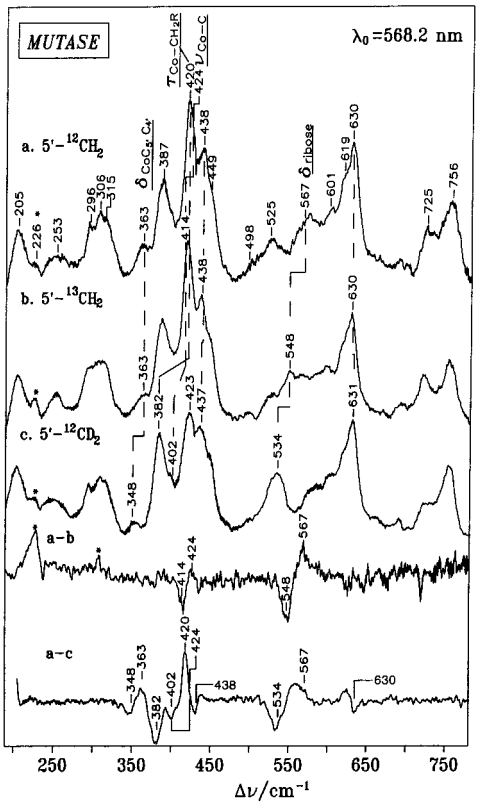

Figure 3. Isotope-edited RR spectra of MMCoA mutase holoenzyme (a). The enzyme was reconstituted with 5′-$^{13}CH_2$ (b) and 5′-$^{12}CD_2$ (c) labeled AdoCbl, and the isotopic spectra were subtracted (a − b, a − c) to isolate the isotope-sensitive modes as difference bands. Conditions: protein concentration = 0.6−0.8 mM; excitation wavelength = 568.2 nm; laser power at the sample = 20 mW; temperature = 77 K; spectral slit width = 3 cm^{-1}. The features marked with * are RR bands of ice.

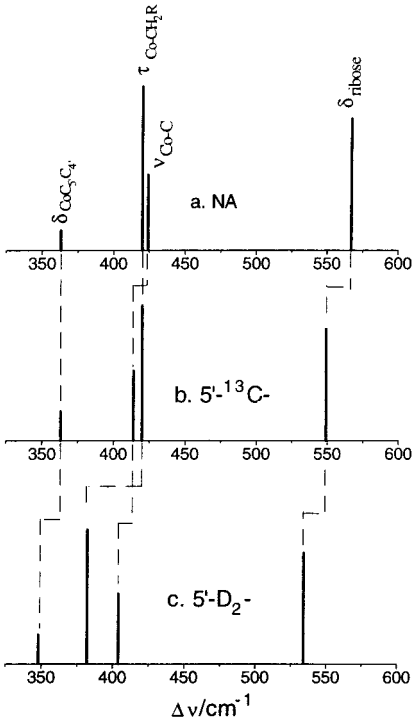

Figure 4. Schematic representation of the Co-Ado modes in MMCoA mutase; the lengths of the bars are proportional to the relative intensities (Table 2).

additional modes, at 420 and 363 cm^{-1}, which show large H/D shifts (38 and 15 cm^{-1}, respectively) but no 5′-^{13}C shifts. The mode at 363 cm^{-1} is assigned to the Co−C−C bending coordinate ($\delta CoC_{5'}C_{4'}$). Alteration of this angle is expected to displace the H atoms on 5′−C (Figure 1), with minimal displacement of the 5′−C atom itself.

What is the origin of the 420 cm^{-1} band? The large H/D shift implies substantial involvement of C−H/D deformation, but all of the standard methylene deformation modes are much higher in frequency than 420 cm^{-1}.[33] We suggest that this band arises from a hindered rotation about the Co−C(adenosyl) bond (τCo−CH_2R); such a rotation would displace the C−H bonds substantially. In addition, it would not displace the 5′−C atom, thereby explaining the striking insensitivity of the band to ^{13}C substitution. Hindered rotations are normally much lower in frequency,[33] but the orientation of the adenosyl ligand is tightly constrained by nonbonded contacts with the corrin ring and its substituents. Thus an elevated frequency is plausible, although there is presently no way to quantitate this effect.

In summary there are four closely spaced Co-adenosyl modes, between 300 and 600 cm^{-1}. These could not have been identified without the use of 5′-^{13}C as well as 5′-CD_2 isotopomers. Two of them, νCoC and τCoH_2R, are nearly coincident with each other and with a corrin mode, so that the strong 420 cm^{-1} band in the natural abundance RR spectrum owes its intensity to three separate contributions. However the corrin bands are cleanly removed in the isotope difference spectra, and the band pattern is clarified by the differential isotope shifts. A schematic diagram is given in Figure 4, and the frequencies for the various adducts are listed in Table 1.

Why are four Co-adenosyl modes enhanced in resonance with a $\pi-\pi^*$ electronic transition of the corrin ring? Enhancement implies coordinate displacement in the resonant excited state. As illustrated in Figure 5, the overlap of the 5′−C sigma orbital with the Co d_{z2} orbital is modulated by interactions with the corrin HOMO.[34] This modulation is affected by stretching of the Co−C bond and also by bending of the Co−C−C angle. These coordinates can therefore be displaced upon electronic excitation from the HOMO to the LUMO. The δribose coordinate is mechanically coupled to the Co−C−C angle and may also be displaced. Finally the nonbonded contacts with the corrin ring determine the Co-Ado hindered rotation frequency, and relief of these contacts in the excited state can therefore produce displacement of $\tau CoCH_2R$.

There are two additional weak sigmoidal features in the 5′-CD_2 difference spectrum (Figure 3), at 438 and 630 cm^{-1}. These result from 1−2 cm^{-1} down- and upshifts of the relatively strong corrin modes at these frequencies. The shifts imply a small degree of coordinate mixing between these corrin modes and 5′-CH_2 deformations.

3. Spectral Changes Upon Substrate and Inhibitor Binding. Isotope editing reveals changes in the adenosyl modes upon substrate and inhibitor binding (Figure 6, 7; Tables 1, 2). As already noted[28] the Co−C stretching frequency shifts from 430 to 424 cm^{-1} when AdoCbl is bound to the apo-enzyme, but it shifts *back* to 430 cm^{-1} when the product, S−CoA (Figure 6), or the substrate, MM-CoA (not shown), is added, although the Co−C intensity is about one-fourth that observed in the free cofactor (Table 2).

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Table 1. Co-adenosyl Mode Frequencies (cm^{-1})

	frequency (cm^{-1})											
	AdoCbl[a]			mutase[b]			mutase + G-CoA[c]			mutase + MMCoA[d]		
vibrations	na[e]	5′-^{13}C[f]	5′-D_2[g]	na	5′-^{13}C	5′-D_2	na	5′-^{13}C	5′-D_2	na	5′-^{13}C	5′-D_2
δ_{ribose}	569	548	534	567	549	534	567	549	534	567	548	534
τ_{Co-CH_2R}	nd	nd	382	420	420	382	420	420	382	420	420	382
ν_{Co-C}	430	420	410	424	414	404	nd	nd	nd	430	420	nd
$\delta_{CoC5'C4'}$	363	363	350	363	363	348	363	363	348	nd	nd	nd

[a] Cofactor in aqueous solution. [b] Holoenzyme. [c] Enzyme plus glutaryl-CoA inhibitor. [d] Enzyme plus methylmalonyl CoA substrate. [e] Natural abundance. [f] ^{13}C in the Ado 5′ position. [g] D_2 on the two Ado 5′ methylene group. ND: not determined.

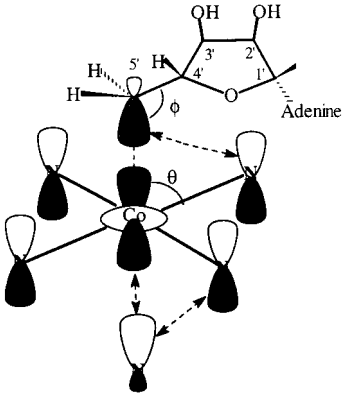

Figure 5. Strain orbital of coenzyme B_{12}, showing the corrin HOMO and the ligand and Co sigma orbitals (adapted from ref 34).

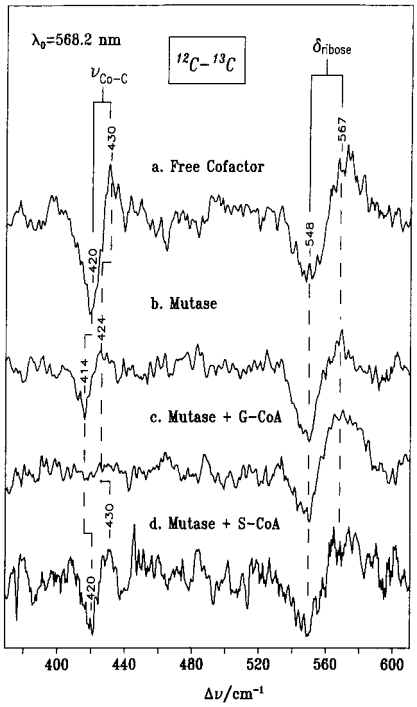

Figure 6. 5′-$^{12}CH_2$ minus 5′-$^{13}CH_2$ difference RR spectra for AdoCbl free in solution (a) and bound to MMCoA mutase (b) in the presence of very slow substrate (G-CoA) (c) and of product (S−CoA) (d). Conditions: Excitation wavelength = 568.2 nm; laser power = 20 mW; enzyme concentration = 0.76 mM (5′-CH_2 enzyme) or 0.86 mM (5′-$^{13}CH_2$ enzyme). For part c, 18 μL of enzyme solution was mixed with 2 μL of 317 mM G-CoA. For part d, 17 μL of enzyme solution was mixed with 3 μL of 134 mM S−CoA.

A different result is obtained when the very slow substrate glutaryl-CoA (G-CoA) is added (Figure 6). νCo−C essentially disappears; we are unable to locate its frequency in the difference spectrum. Likewise νCo−C cannot be located when the inhibitor CPC−CoA, or the slow substrate EtM-CoA, is added to the holoenzyme (data not shown). EtM-CoA and G-CoA react slowly enough (hours to days) that rearrangement to product is insignificant during the two minutes it takes to mix and freeze the solutions for RR spectroscopy. All three modified substrate structures (Chart 1) eliminate the τCo−C signal upon binding to the holoenzyme. However, the δribose mode does not shift in any of the AdoCbl preparations, although its intensity diminishes in the enzyme, and especially upon substrate addition (Table 1). This signal ensures that the Ado ligand remains bound to the Co under all conditions.

When the CH_2/CD_2 difference spectra (Figure 7) are examined, the τCoCH$_2$R bands (420/382 cm^{-1}) are seen to be

Table 2. Relative Intensity Variations among the Ado Ligand Modes[a]

vibrations	AdoCbl[b]	mutase	mutase + G-CoA	Mutase + MMCoA
δ_{ribose}	3.3	1.9	2.4	1.0[a]
τ_{CoCH_2R}	2.1	2.3	2.2	2.2
ν_{Co-C}	3.9	1.2	0	0.9
$\delta_{CoC5'C4'}$	~0	0.5	1.2	0

[a] The 567 cm^{-1} peak intensity in enzyme + MMCoA is taken as 1. The relative intensity is the ratio of the peak height between the peak and valley of the sigmoidal feature in the difference spectra. [b] Peak intensities of the cofactor are related to those of the enzyme by normalizing the 630 cm^{-1} corrin peak.

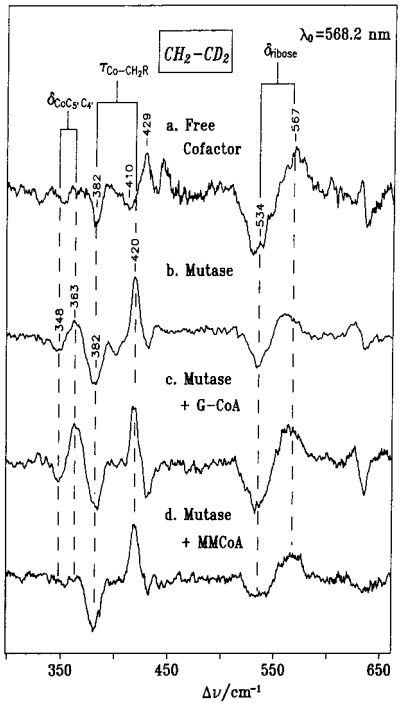

Figure 7. As Figure 7, for 5′-CH_2 minus 5′-CD_2 spectra, except MMCoA was used instead of S−CoA in part d and 3 -D_3-MMCo was used to prevent isotope washout of the 5′-CD_2 spectrum.

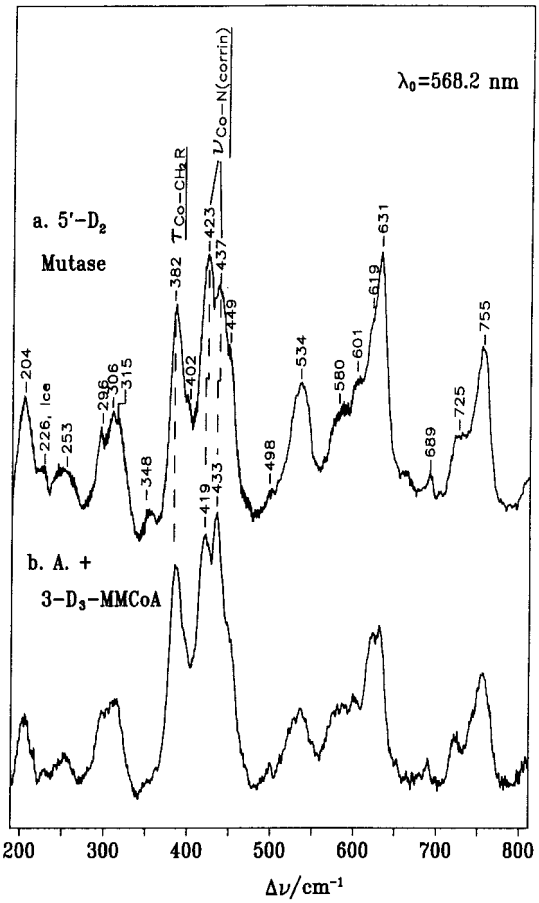

Figure 8. Low-frequency RR spectra for 5′-CD_2-labeled MMCoA mutase, showing shifts in two corrin bands upon adding 3-CD_3-labeled substrate.

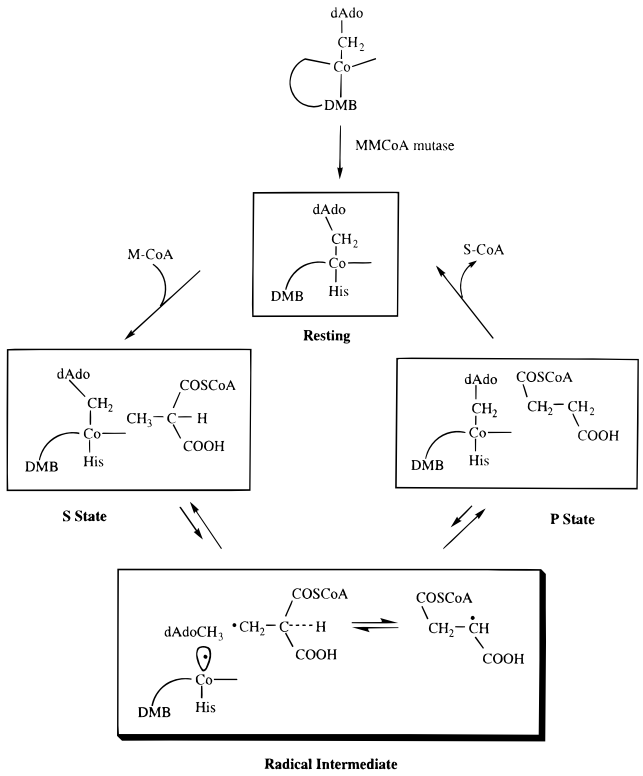

Figure 9. Proposed scheme for Co−C bond activation in MMCoA mutase. The Ado ligand is tilted, and the Co−C bond is weakened slightly in the resting enzyme. Substrate binding induces a protein conformation change[17] and increases Co-Ado tilting (S state). Rearrangment to product, via transiently generated radical intermediates, restores the Ado ligand and deactivates the Co−C bond in the product complex (P state). The DMB axial base is displaced by a histidine residue, and the Co−N(His) bond is stretched,[17] at least in the Co(II) form, and possibly also in the P state (see text).

unaltered in any of the preparations. (For the free cofactor, the τCoCH_2R band in the CH_2 spectrum is a shoulder on the much stronger νCo−C mode, but the negative τCoCD_2R band in the CD_2 spectrum is as strong as in the protein spectra: see Table 2). However, the δCoCC mode varies strongly in intensity. It is barely detectable in the free cofactor but intensifies in the holoenzyme and doubles in strength when the very slow substrate G-CoA is added. The same intensification is seen when CPC−CoA is added (not shown). However, when MM-CoA is added to the holoenzyme, the δCoCC band disappears (Figure 7d). This last experiment required an addition of 3-D_3-MM-CoA, in order prevent washout of the CD_2 spectrum via isotope exchange. (Isotope exchange was also found to wash out the CD_2 spectrum of EtM-CoA. Thus, the CH_2/CD_2 difference spectrum was unobtainable for EtMCoA, since the appropriately deuterated isotopomer was unavailable.)

The use of 3-D_3-MM-CoA with 5′-CD_2 cofactor also permitted the observation of distinct downshifts in two corrin modes, at 423 and 437 cm^{-1}, when substrate is added to the holoenzyme (Figure 8). Without deuteration, this effect is obscured by the 420 cm^{-1} band. The corrin bands are unshifted when G-CoA or CPC−CoA is added to CD_2 holoenzyme. The composition of these corrin modes has not been determined, but their frequencies suggest significant Co−N(corrin) stretching contributions. Thus addition of substrate, but not of inhibitor, appears to weaken the Co−N(corrin) bonds somewhat.

In summary, binding of AdoCbl to MM-CoA mutase lowers the Co−C stretching frequency and weakens the RR band, while intensifying the CoCC bending mode. This trend is augmented by inhibitor or slow substrate binding, which doubles the δCoCC intensity and diminishes the νCo−C intensity to undetectability. However, the trend is *reversed* by substrate or product binding, which restores νCoC to the frequency (though not the intensity) seen in the free cofactor and eliminates δCoCC. In addition the Co−N(corrin) bonds may be weakened by substrate/product but not by inhibitor/slow substrate binding.

Discussion

1. Evidence for Separate Substrate and Product States. The crystal structures of Evans and co-workers[17] establish a large-scale conformation change when MMCoA mutase binds substrate analogues. The two halves of the $(\beta\alpha)_8$ TIM barrel close up around the substrate, burying it from solvent. This conformation change is essentially the same for the different substrate analogues examined crystallographically, but the adenosyl group is missing in all of these structures and the cobalamin appears to contain Co(II).

In the RR experiments, the Ado ligand is intact and quite different spectral patterns are observed, depending on whether the enzyme contains substrate or product on the one hand or slow substrates or inhibitors on the other. To explain this dichotomy in the observed spectral changes, we propose that the enzyme can exist in three separate states (Figure 9), the resting state, an S state, and a P state. The S state is associated with substrate binding, while the P state is associated with product binding. However, the S state is normally unpopulated because the substrate is rapidly rearranged to product. Only

when the rearrangement is slowed or prevented can the S state be observed. The S state promotes Co−C bond homolysis followed by H-atom transfer between substrate and adenosyl radical and rearrangement of the substrate radical to product radical.[35] These radical intermediates are present at low concentrations. EPR signals have amplitudes corresponding to no more than 20% of that expected for complete Co−C homolysis.[29] Product is formed by H atom abstraction from adenosine and reformation of the Ado-Co bond. The P state accumulates because product dissociation is apparently the rate-limiting step in the overall enzyme reaction[37]and because the equilibrium between MM-CoA and S−CoA favors S−CoA by 20:1.[36,37] Since rearrangement is complete within the two minutes between mixing and freezing of the sample, we obtain the P state RR spectra whether substrate or product is added to MM-CoA mutase.

It is the substrate analogues that permit observation of the S state because rearrangment is slowed. In the case of EtM-CoA rearrangment is slowed by 10^{3-4}, relative to MM-CoA.[38,39] The extent of rearrangement is negligible on the time scale of mixing and freezing the sample. Nevertheless EtM-CoA does induce rapid 5′-CH_2-AdoCbl exchange; the 5′-CD_2 RR spectrum was washed out within the mixing time. This behavior is consistent with the observation of Retey and co-workers[39] that an EPR signal characteristic of Co[II] coupled to an organic radical is produced in the frozen steady state of EtM-CoA-bound mutase. Thus EtM-CoA produces a productive complex with respect to Co−C homolysis, but rearrangement of the EtM-CoA radical is slowed, permitting spectroscopic characterization of the S state. The RR spectrum is the same as that obtained with G-CoA (Figure 6); the Ado ligand is intact, as revealed by δribose, but the νCo−C band is undetectable.

G-CoA is a very slow substrate (days required for rearrangement),[40] and in this case, we do not observe washout of the 5′-CD_2 spectrum in the two minutes before the sample is frozen. Thus we were able to obtain a CH_2/CD_2 difference spectrum which reveals intensification of the δCoCC band (Figure 7). The same spectral changes are observed upon addition of CPC−CoA, for which no rearrangement is detectable.[30]

We infer that all three substrate analogues produce the S state, but they are unable to proceed to the P state. In the case of EtM-CoA, rapid 5′-CH_2/D exchange is observed, showing the complex to be productive. This exchange is not observed for G-CoA and CPC−CoA during the mixing time. It is likely that these analogues also promote Co−C homolyis but that the 5′-CH_2/D exchange reaction is inhibited because of incorrect positioning of the relevant C−H bonds (Chart 1).

3. CoC Activation Mechanism. a. Steric Repulsion by the Corrin. The RR data do not support the idea that the Co−C bond is activated by ground-state enzyme-induced steric repulsion between the corrin ring and the bulky adenosyl ligand. This repulsion already exists in the free cofactor and lowers the force constant by 20%,[20] relative to MeCbl, whose methyl ligand offers much less steric hindrance. The bond dissociation energy (BDE; 37 ± 3 kcal/mol for MeCbl[41] and 31.5 ± 2 kcal/mol for AdoCbl[5]) is reduced by the same percentage, consistent with a ground-state effect resulting from the steric strain of the bulky Ado ligand. The steric bulk of the Ado ligand may be why nature has chosen the Ado ligand for reactions requiring radical generation via Co−C homolysis.[3]

Nonbonded contacts are evident in the AdoCbl crystal structure, which reveals a butterfly fold of the corrin ring, with wings bent toward the Ado ligand.[42] However, binding the cofactor to the enzyme flattens the corrin ring. The MMCoA mutase crystal structures reveal a more planar corrin conformation,[17] and the high-frequency corrin RR bands shift up upon binding AdoCbl to the mutase, the direction expected if ring distortion is lessened.[28] Addition of substrate or inhibitors produces no additional changes in the corrin ring modes, (Figure 2) showing that the corrin conformation is the same in the S and P states as in the resting state. Thus, the corrin ring is not directly involved in the Co−C activation mechanism.

b. Activation by CoC Tilting. The RR data provide direct evidence that Co−C tilting is part of the activation mechanism. In the free cofactor, νCo−C is enhanced but δCoCC is not. This means that the electronic excitation (Figure 5) lengthens the Co−C bond but does not alter the CoCC angle. However δCoCC is activated when AdoCbl is bound to the mutase, and it is further intensified in the S state. At the same time, the νCo−C progressively diminishes. These observations suggest that the Co−C bond is tilted by interactions of the Ado ligand with the residues in the enzyme binding pocket and that this tilting is increased upon substrate (or analog) binding. As a result, the Co−C bond is weakened, and its extension upon electronic excitation is diminished, thereby reducing the νCo−C intensity. At the same time, the sterically induced Co−C tilting is reduced upon electronic excitation, and δCoCC is consequently displaced between the ground and excited state, producing RR enhancement. The intensity shift from νCo−C to δCoCC is a manifestation of Co-Ado angular distortion, induced by the constraints of the enzyme and of the substrate.

Angular distortion is an effective mechanism for Co−C bond weakening, as judged from orbital overlap considerations.[14,33] Bond weakening in the ground state can be estimated from the Co−C frequency. Thus the 6 cm^{-1} decrease in νCo−C upon binding the cofactor to the resting enzyme corresponds to a 0.5 kcal/mol weakening of the bond, if it is assumed that the bond energy is proportional to the force constant and that the latter is proportional to the square of the frequency.[21] The extent of Co−C activation in the resting enzyme is small, as expected. The Co−C bond is certainly weakened further in the S state, but unfortunately, we are unable to estimate the extent because νCo−C cannot be located.

Arrestingly, the Co−C bond appears to be *deactivated* in the P state. The RR spectra reveal no enhancement of δCoCC, and the Co−C stretching frequency, 430 cm^{-1}, is fully as high as it is in the free cofactor. We conclude that the forces which induce Co-Ado distortion in the S state are relieved when the substrate rearranges to product on the enzyme. The resulting deactivation of the Co−C bond must lower the rate of the back reaction, thereby promoting product accumulation.

The angular distortion of the Ado ligand must be due to steric crowding by the substrate and by the surrounding protein. This crowding is relieved in the P state. Since the crystal structures show essentially the same atomic positions for the protein whether substrate or product analogues are bound,[17c] it seems unlikely that the S and P states have different protein conforma-

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tions. Relief of the steric crowding may result from the altered shape of the rearranged product (see Figure 9).

c. Status of the Axial Base. A much remarked feature of MMCoA mutase,[17] as of methionine synthase,[18] is the displacement of the DMB axial base (Figure 1) with a histidine side chain in the enzyme. What is more, the Co−N(His) bond in MM-CoA mutase was found to be very long, 2.5 Å.[17]

The functional significance of this trans bond lengthening is currently uncertain. It was suggested[17] that the long Co−N bond might stabilize the Co[II] species relative to Co[III], thus favoring adenosyl radical formation. However for the free cofactor, the Co−N bond to the trans DMB ligand is actually longer in the Co[III]-Ado form, 2.21 Å,[43] than in the Co[II] form, 2.13 Å.[44] The abnormally long trans bond in the Co[III]-Ado form reflects the well-known ground-state trans effect of alkyl ligands.[45] The trans effect is large enough that further lengthening of the trans bond would appear to favor Co[III]-Ado over Co[II].

However, De Ridder et al.[45] find that a secondary inverse trans effect is superimposed on this primary trans effect. From an analysis of crystallographic data on many cobalamin and cobaloxime complexes, they find that the trans Co−C and Co−N bond distances are *positively* correlated: as one increases, so does the other. This positive cooperativity implies that weakening the Co−N bond weakens the Co−C bond. Indeed, Halpern's classic measurements of cobaloxime Co−C bond strengths[4] are in accord with this expectation: the Co−C strength diminishes with decreasing basicity of the trans (substituted pyridine) ligand. From this evidence, the Co-Ado bond is expected to weaken if the Co−N(His) bond in MM-CoA mutase is lengthened by protein forces.

However, Sirovatka and Finke[9] find that binding *N*-methylimidazole to adenosylcobinamide (AdoCbl with the axial base cleaved off chemically) *increases* the rate of Co−C homolysis, by a factor of 870 relative to the solvent (ethylene glycol) as trans ligand and by a factor of 8 relative to dimethylbenzimidazole in AdoCbl. *N*-Methylimidazole is more basic than dimethylbenzimidazole, which is more basic than ethylene glycol, so these observations run counter to the inverse trans effect. It is possible that the latter operates only over a limited range of trans ligand alterations. Thus the significance of the long Co−N(His) bond with respect to Co−C activation remains uncertain.

A long axial bond could explain our observation that the 423 and 427 cm^{-1} corrin modes are selectively downshifted upon substrate addition (Figure 8). These shifts could be explained by Co−N(corrin) bond weakening as a result of displacement of the Co atom toward the Ado ligand when the trans Co−N(His) bond is lengthened. These shifts are not observed in the adducts with G-CoA and CPC−CoA, suggesting the possibility that Co−N(His) bond lengthening occurs in the P state but not in the S state. If this is the case, then Co−N(His) bond weakening helps *deactivate*, not activate, the Co−C bond, consistent with the primary trans effect. However this hypothesis requires further testing, since the proposed connection between the two corrin modes and the Co−N(His) bond is speculative.

A more clear-cut role for the long Co−N(His) bond emerges from the study of Sirovatka and Finke,[9] who find that binding *N*-methylimidazole to adenosylcobinamide strongly accelerates Co−C bond *heterolysis*, much more than homolysis. Heterolysis accounts for 50% of the overall Co−C cleavage when *N*-methylimidazole is the trans ligand, but only 2% for trans dimethylbenzimidazole in AdoCbl. The heterolysis reaction involves an internal fragmentation of the Ado ligand leaving Co[III]Cbl. In the absence of the trans-directing alkyl group, Co[III] is strongly stabilized by a more basic trans ligand. Weakening the bond to the base would therefore destabilize the Co[III] product, and *inhibit* heterolysis, as Sirovatka and Finke point out. Thus the protein-induced Co−N[His] lengthening may primarily be a mechanism for preventing a destructive side reaction.

Conclusions

Isotope-edited RR spectra give evidence for Co−C tilting as part of the Co−C activation mechanism in MM-CoA mutase. The RR enhancement patterns clearly establish δCoCC intensification and νCo−C weakening in the holoenzyme, which are accentuated by binding inhibitor or slow subtrates (S state). This pattern is reversed when the enzyme contains bound substrate or product (P state), indicating that the steric forces responsible for the Co−C tilting are relieved upon rearrangement of substrate to product at the active site. The absence of change in most of the corrin ring modes indicates that the corrin conformation, which is flattened in the enzyme relative to free cofactor, is unaltered by substrate or inhibitor binding and does not contribute to the activation mechanism. However, substrate binding does selectively lower two low-frequency corrin modes, possibly due to Co displacement from the corrin plane. This motion could result from weakening of the Co−N(His) bond, as is observed in the crystal structure of the Co[II] form of the enzyme. The effect of this weakening on the Co−C bond is still uncertain, but it plays an important role in preventing Co−C heterolysis.

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