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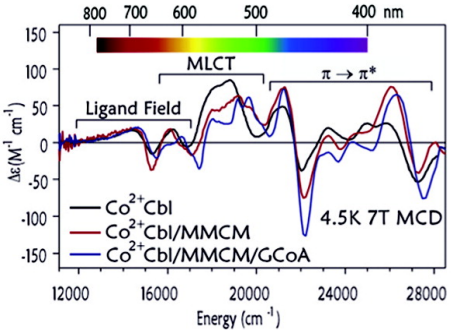

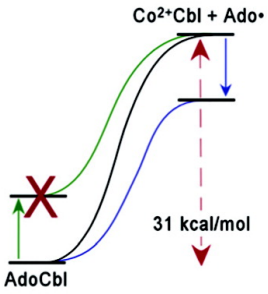

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Co−C Bond Activation in Methylmalonyl-CoA Mutase by Stabilization of the Post-homolysis Product Co^{2+}Cobalamin

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Abstract: Despite decades of research, the mechanism by which coenzyme B_{12} (adenosylcobalamin, AdoCbl)-dependent enzymes promote homolytic cleavage of the cofactor's Co−C bond to initiate catalysis has continued to elude researchers. In this work, we utilized magnetic circular dichroism spectroscopy to explore how the electronic structure of the reduced B_{12} cofactor (i.e., the post-homolysis product Co^{2+}Cbl) is modulated by the enzyme methylmalonyl-CoA mutase. Our data reveal a fairly uniform stabilization of the Co 3d orbitals relative to the corrin π/π^*-based molecular orbitals when Co^{2+}Cbl is bound to the enzyme active site, particularly in the presence of substrate. Contrastingly, our previous studies (Brooks, A. J.; Vlasie, M.; Banerjee, R.; Brunold, T. C. *J. Am. Chem. Soc.* **2004**, *126*, 8167−8180.) showed that when AdoCbl is bound to the MMCM active site, no enzymatic perturbation of the Co^{3+}Cbl electronic structure occurs, even in the presence of substrate (analogues). Collectively, these observations provide direct evidence that enzymatic Co−C bond activation involves stabilization of the post-homolysis product, Co^{2+}-Cbl, rather than destabilization of the Co^{3+}Cbl "ground" state.

Introduction

Methylmalonyl-CoA mutase (MMCM) is the only coenzyme B_{12} (adenosylcobalamin, AdoCbl)-dependent enzyme found in humans,[1] where it catalyzes the conversion of methylmalonyl-CoA (MMCoA) to succinyl-CoA (SCoA).[2] The AdoCbl cofactor (Figure 1) plays an integral role in this radical rearrangement reaction, as catalysis is initiated through homolytic cleavage of its Co−C bond to yield Co^{2+}Cbl and an adenosyl radical that is poised to abstract a hydrogen atom from the substrate (Scheme 1).[2] A particularly fascinating aspect of this reaction is that the rate of homolytic Co−C bond cleavage shows a spectacular degree of enhancement when the cofactor is bound in the enzyme active site, increasing by as much as 10^{12}-fold over that of the free cofactor.[3,4] Two conceivable pathways by which this rate enhancement can occur are (a) destabilization of the AdoCbl "ground state" and (b) stabilization of the post-homolysis product Co^{2+}Cbl.[5] Previous research carried out by us[6] and in collaboration with Spiro and co-workers[7,8] has indicated that the former pathway contributes little to the ∼17 kcal/mol reduction in Co−C bond strength required by the enzyme[4] to accomplish the trillion-fold acceleration of Co−C bond homolysis. In support of the latter pathway, we report here

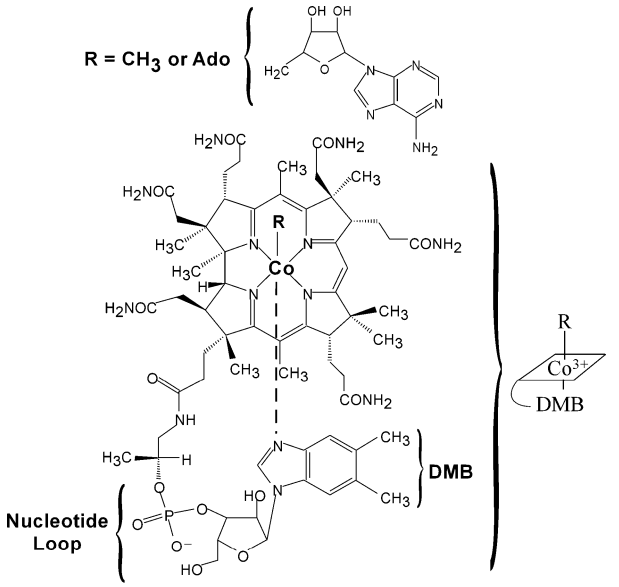

Figure 1. Chemical structure of MeCbl (R = CH_3), AdoCbl (R = Ado), and Co^{2+}Cbl (lacking an R group).

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(5) For bond homolysis reactions, the combined energies of the two nascent radical species (or, as in the present case, an Ado• radical and Co^{2+}Cbl) will be very close to the energy of the transition state according to Hammond's postulate. Stabilization of the post-homolysis product will therefore result in a lowering of the activation barrier and a consequent increase in the reaction rate. Thus, for AdoCbl-dependent enzymes it is reasonable to correlate an increase in the Co−C bond homolysis rate constant with a reduction in the relative energies of the AdoCbl "ground state" and the post-homolysis products Co^{2+}Cbl and Ado•.

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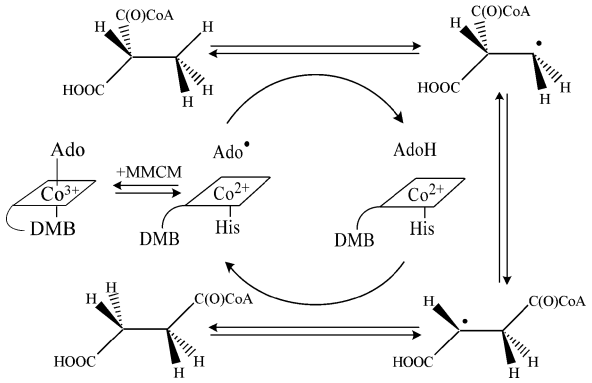
Scheme 1. Minimal Mechanism for the Interconversion of Methylmalonyl-CoA (MMCoA, top left) and Succinyl-CoA (SCoA, bottom left) by MMCM



spectroscopic evidence that the Co^{2+}Cbl cofactor is stabilized by the enzyme active site and that this stabilization is enhanced by the presence of substrate (analogues).

Previous spectroscopic investigations of Co^{2+}Cbl bound to the MMCM active site have primarily relied on electronic absorption (Abs) and electron paramagnetic resonance (EPR) techniques.[9−12] The inherent presence of a mixture of AdoCbl and Co^{2+}Cbl in MMCM samples trapped under steady state turnover conditions, however, has greatly limited the value of Abs spectroscopy in probing enzymatic perturbations of the Co^{2+}Cbl cofactor.[11] Alternatively, although EPR spectroscopy provides a selective probe of Co^{2+}Cbl, this technique is sensitive only to axial perturbations of the cofactor, as the single unpaired electron resides in a Co d_{z^2}-based molecular orbital (MO) that contains little contribution from the equatorial nitrogens of the corrin macrocycle.[13]

As revealed by our study of free Co^{2+}-corrinoids,[13] a technique that potentially overcomes these shortcomings is magnetic circular dichroism (MCD). While both EPR and low-temperature MCD spectra are dominated by contributions from paramagnetic species,[14] the MCD technique has the added benefit of yielding insight into both axial and equatorial perturbations of the cobalt center of Co^{2+}Cbl while simultaneously also reporting on the geometric and electronic properties of the corrin ring,[6,15] thus offering an almost ideal probe of the interactions between Co^{2+}Cbl and the MMCM active site.

To explore the nature of these cofactor/enzyme interactions, we examined the electronic structure of Co^{2+}Cbl bound to the MMCM active site both in the absence and the presence of substrate (analogues) using MCD spectroscopy. Significant enzymatic perturbations of the electronic structure of Co^{2+}Cbl, particularly in the presence of substrate (analogues), are indicated by notable changes in the spectroscopic signatures. Interpretation of these changes within the framework of density functional theory (DFT) and time-dependent DFT (TD-DFT) calculations leads to the conclusion that the Co−C bond activation mechanism involves enzymatic manipulation of the post-homolysis product Co^{2+}Cbl and occurs via stabilization of the Co 3d-based molecular orbitals.

Experimental Details

Chemicals/Cofactors. Adenosylcobalamin (AdoCbl), methylcobalamin (MeCbl), aquacobalamin (H_2OCbl^+), methylmalonyl-CoA (MMCoA), and glutaryl-CoA (GCoA) were purchased from Sigma and used as obtained. Ethylmalonyl-CoA (EtMCoA) was synthesized as described previously.[6,16] The substrates were dissolved in potassium phosphate buffer at pH 7.5 to a concentration of 50 mM. Co^{2+}Cbl was generated by reduction of H_2OCbl^+ using the flavin system from *Salmonella enterica*, as described previously.[13,17,18]

Purification of Methylmalonyl-CoA Mutase (MMCM). The recombinant *Propionibacterium shermanii* enzyme expressed in *Escherichia coli* was purified through the step preceding reconstitution with the cofactor as previously described.[11] Protein concentration was determined by the Bradford method using bovine serum albumin as a standard. Reconstitution of apoenzyme with AdoCbl and MeCbl was achieved as previously described.[6] Unbound cofactor was subsequently removed by gel filtration chromatography on a Micro Bio-Spin P-30 (Bio-Rad) column.

Sample Preparation. Samples used for low-temperature experiments were prepared in 60% (v/v) glycerol glassing agent. All sample solutions were purged with N_2 gas for 15 min before being injected into MCD sample cells and frozen in liquid nitrogen. To generate MMCM-bound Co^{2+}Cbl, apo-MMCM was reconstituted with MeCbl and exposed to direct light from a 60-W bulb at ∼6 in. while in the MCD sample cell. After 45 min, approximately 60% of the cofactor was photolyzed to yield Co^{2+}Cbl/MMCM, at which point the sample was frozen in liquid N_2. Substrate (analogue)-bound holo-MMCM samples were obtained as described previously[6] by the addition of at least a 40-fold molar excess of the appropriate substrate (analogue) to apo-MMCM that had been reconstituted with either AdoCbl or MeCbl. These samples were then exposed to room light for up to several hours to promote formation of the Co^{2+}Cbl/MMCM/substrate complex. Conversion to Co^{2+}Cbl was monitored by Abs spectroscopy and was achieved successfully for AdoCbl/MMCM with GCoA and for MeCbl/MMCM with MMCoA, EtCoA, and GCoA. Upon conversion to an adequate percentage (∼30−60%) of Co^{2+}Cbl, the samples were frozen in liquid N_2. Catalytic turnover for MMCM reconstituted with the native cofactor AdoCbl in the presence of MMCoA and EtMCoA occurred too quickly for the Co^{2+}Cbl-bound intermediate state to be trapped on the time scale of this sample preparation.

Spectroscopy. Electronic Abs and MCD spectra were obtained using a Jasco J-715 spectropolarimeter in conjunction with an Oxford Instruments SM-4000 8T magnetocryostat. Cofactor concentrations were determined spectrophotometrically at 300 K on the basis of published molar extinction coefficients[19] and ranged from 0.1 to 0.4 mM. All MCD spectra reported in this paper were obtained by subtracting the −7 T spectrum from the 7 T spectrum to eliminate contributions from the natural CD.

Computations. All cofactor models were constructed on the basis of crystallographic data reported for Co^{2+}Cbl[20] and MeCbl[21] using the

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truncation scheme developed previously in our laboratory for Co^{3+}-Cbls and Co^{2+}Cbls.[13,15] Atomic coordinates for these models can be found in the Supporting Information (Tables S1 and S2).

Spin-unrestricted DFT calculations on the Co^{2+}Cbl model were carried out using both the Amsterdam Density Functional (ADF) 2003.01 suite of programs[22−24] and the ORCA 2.2 software package.[25] The single-point DFT calculation with ADF was performed utilizing the Vosko−Wilk−Nusair local density approximation (VWN-LDA)[26] with the nonlocal gradient corrections of Becke[27] for exchange and Perdew[28] for correlation, ADF basis set IV, and an integration constant of 4.0. Core orbitals were frozen through 1s for C, N, and O and 2p for Co. Following this single point calculation, additional DFT computations were carried out to estimate electronic transition energies using the half-electron excitation method of Slater,[29] where the difference in orbital energies at convergence after promotion of half of an electron from the donor molecular orbital (MO) to the acceptor MO is taken as the electronic transition energy. The ORCA single-point DFT calculation employed the Perdew−Wang LDA[30] with gradient corrections by Becke[27] and Perdew[28] in conjunction with the DGauss (Gaussian polarized double-ζ valence orbital) basis[31] and the Demon/J auxiliary basis.[31] MO energies and compositions obtained from the DFT calculations using both software packages were virtually identical. Therefore, the output provided by the latter program could be used in conjunction with Laaksonen's gOpenMol program[32,33] to generate isosurface plots of relevant MOs involved in the electronic transitions whose energies were estimated by the method of Slater[29] using the former program (note that only ADF allows for the fractional occupation of MOs as required by the Slater half-electron excitation method).

Additional calculations were carried out with both ORCA and ADF utilizing the conductor-like screening model (COSMO) of solvation[34−38] to evaluate the effects of different dielectric environments on the calculated transition energies. In ORCA, the default values for the atomic radii (1.2 times the van der Waals surface) were used to construct the molecular surface of the solute (2.223 Å for Co, 1.83 Å for N, 2.0 Å for C, and 1.30 Å for H). TD-DFT calculations were carried out as above for Co^{2+}Cbl and MeCbl surrounded by COSMO solvation fields with either a high or a low dielectric constant (ϵ = 80 or 10, respectively) and a refractive index of 1.33. In ADF, single-point DFT calculations were carried out as above for the Co^{2+}Cbl model in the presence of a COSMO field with either ϵ = 80 or ϵ = 10 using the same atomic radii given above for the ORCA calculations. New energies for specific electronic transitions were then computed with ADF by taking the difference in the orbital energies following the excitation of half an electron from the donor MO to the acceptor MO in the presence of the COSMO solvation fields.

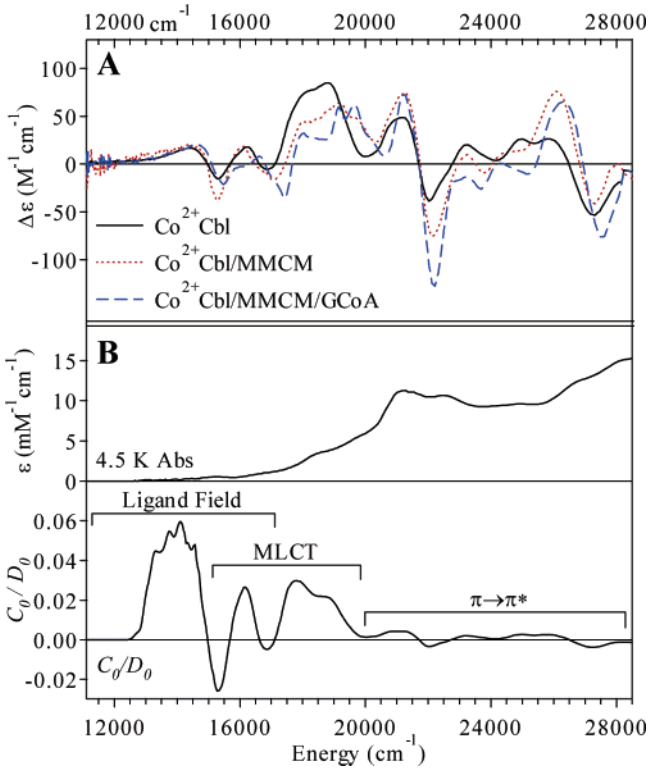

Figure 2. (A) 4.5 K, 7 T MCD spectra of free Co^{2+}Cbl, MMCM-bound Co^{2+}Cbl (Co^{2+}Cbl/MMCM), and MMCM-bound Co^{2+}Cbl in the presence of the slow substrate glutaryl-CoA (Co^{2+}Cbl/MMCM/GCoA). (B) 4.5 K Abs spectrum (top) and C_0/D_0 plot[41] (bottom) for free Co^{2+}Cbl with general band classifications indicated above the C_0/D_0 plot.

Results and Analysis

Spectroscopy. Figure 2A shows a superposition of MCD spectra obtained for (i) aqueous Co^{2+}Cbl, (ii) MMCM-bound Co^{2+}Cbl, and (iii) MMCM-bound Co^{2+}Cbl in the presence of the slow substrate glutaryl-CoA (GCoA). The MCD spectra of MMCM-bound Co^{2+}Cbl in the presence of the actual substrate methylmalonyl-CoA (MMCoA) and the substrate analogue ethylmalonyl-CoA (EtMCoA) are virtually identical to the Co^{2+}-Cbl/MMCM/GCoA spectrum shown in Figure 2A; a comparison of these three spectra is presented in Figure 3.[39] For each of the three samples used to obtain the spectra in Figure 3, Co^{2+}-Cbl was generated via photolysis of holo-MMCM in the presence of the appropriate substrate (analogue) to increase the Co^{2+}Cbl/Co^{3+}Cbl ratio over that obtained under turnover conditions. Although we have no direct evidence that the Co^{2+}-Cbl species present in these samples is catalytically relevant, this is a reasonable assumption in light of the fact that the MMCM active site is deeply buried and, consequently, release of the Ado group is not expected to occur during photolysis under the conditions employed in this study. Note that evidence for retention of the other dissociable group in the MMCM active site, the substrate molecule, is provided by the MCD spectra presented in Figure 2. In further support of this assumption, recent studies carried out in our laboratory have shown that the

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(39) The MCD spectrum of the GCoA-bound Co^{2+}Cbl/MMCM sample is presented in Figure 2 instead of that obtained for the MMCoA-bound Co^{2+}-Cbl/MMCM sample as the former exhibited the highest conversion rate to Co^{2+}Cbl upon photolysis and thus yielded data with the best signal/noise ratio.

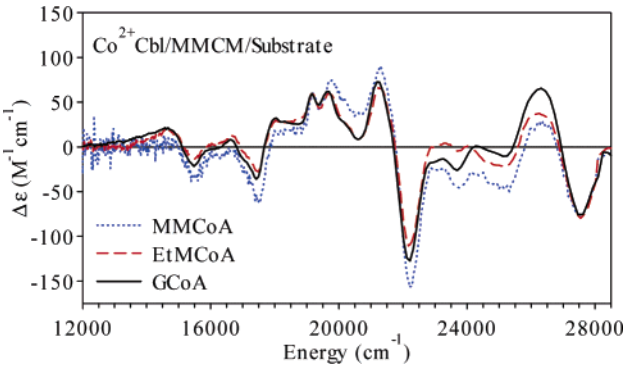

Figure 3. 4.5 K, 7 T MCD spectra of Co^{2+}Cbl/MMCM in the presence of the native substrate methylmalonyl-CoA (MMCoA) and the substrate analogues ethylmalonyl-CoA (EtMCoA) and glutaryl-CoA (GCoA).

spectroscopic signatures of the substrate-bound form of Co^{2+}-Cbl/MMCM are virtually identical to those associated with Co^{2+}-Cbl generated in the glutamate mutase (GM) active site in the presence of substrate during catalytic turnover, that is, with both the substrate and the Ado moiety residing in the enzyme active site.[40]

All of the samples included in this study, except that of the unbound Co^{2+}Cbl cofactor, contained a mixture of Co^{3+}Cbl ($\geq$40%) and Co^{2+}Cbl ($\leq$60%). As both species exhibit similarly intense electronic transitions and thus contributed significantly to our Abs spectra, this technique failed to provide conclusive information regarding alterations to the post-homolysis product Co^{2+}Cbl in the MMCM active site. The MCD technique, however, due to its particular sensitivity to paramagnetic species, permitted us to selectively probe the fraction of MMCM-bound Co^{2+}Cbl in our samples. Specifically, at 4 K the temperature-independent B-terms originating from unreacted diamagnetic AdoCbl and MeCbl are at least 10 times less intense than the temperature-dependent C-term features associated with paramagnetic Co^{2+}Cbl.[13,15] To make certain that differences in the various Co^{2+}Cbl MCD spectra were not artifacts of the remaining AdoCbl or MeCbl, any weak contributions from the Co^{3+}Cbls were subtracted from the original spectra after fitting the corresponding Abs spectra to determine the Co^{2+}Cbl/Co^{3+}-Cbl ratio. The resulting difference spectra (shown in Figures 2A and 3) were then rescaled so as to reflect 100% occupation of the enzyme active site by Co^{2+}Cbl to facilitate comparison between the different species investigated.

Spectral Analysis. Free Co^{2+}Cbl. Although the Abs and MCD spectra of Co^{2+}Cbl have been described in detail previously,[13] a basic understanding of the electronic structure of the free cofactor is required to adequately interpret spectral changes accompanying (i) Co^{2+}Cbl binding to the MMCM active site and (ii) the addition of substrate (analogues) to the holoenzyme. The complementary nature of the selection rules governing the intensities of electronic transitions in the Abs and MCD spectra permits classification of the Co^{2+}Cbl spectral features according to their electronic origins. This is accomplished by taking the ratio of the MCD and Abs spectra (Figure 2, parts A and B, respectively) and appropriate scaling to obtain a so-called C_0/D_0 plot.[41] In general, $|C_0/D_0|$ values greater than ~0.05 are indicative of ligand field (LF) transitions, whereas values in the ~0.01−0.05 range and below ~0.01 signify charge transfer (CT) and ligand centered (corrin $\pi\rightarrow\pi^*$) transitions, respectively.[14] Ligand field (d→d) transitions carry little Abs intensity, as they are formally parity forbidden but exhibit large C_0/D_0 ratios because of their ability to acquire significant MCD intensity through the spin−orbit coupling mechanism. Hence, the electronic transitions in Co^{2+}Cbl responsible for the prominent MCD features below 16 000 cm^{-1}, which carry very little Abs intensity, can confidently be assigned as LF transitions. Similarly, ligand-centered transitions primarily involving MOs of the corrin π system are easily identified between 20 500 and 28 500 cm^{-1} on the basis of their very low C_0/D_0 ratios (<0.005) and high ϵ values (>10 mM^{-1} cm^{-1}) in the Abs spectrum. Indeed, the dominant Abs feature in the visible region at ~21 100 cm^{-1} has previously been assigned to the lowest energy corrin $\pi\rightarrow\pi^*$ transition, corresponding to the transition responsible for the so-called α-band in the Abs spectra of free Co^{3+}Cbls.[6,13] The remaining transitions that contribute to the MCD spectrum of unbound Co^{2+}Cbl between 16 000 and 20 500 cm^{-1} have intermediate C_0/D_0 values and are thus ascribed to transitions possessing considerable Co 3d → corrin π^* CT (MLCT) character.

To verify these qualitative band assignments and to identify the specific donor and acceptor MOs involved in the corresponding transitions, DFT and TD-DFT calculations were carried out on a suitably truncated model of Co^{2+}Cbl. While the TD-DFT results obtained in this study and our earlier investigation of free Co^{2+}-corrinoids[13] generally corroborate the qualitative band assignments provided above, the heavily mixed nature of the transitions contributing to the computed Abs spectrum (due primarily to the similar energies of the Co 3d and the corrin macrocycle frontier orbitals) made specific band assignments difficult. Therefore, the method of half-electron excitation developed by Slater[29] was used to obtain a direct estimate of the energies of electronic transitions involving a specific pair of donor and acceptor MOs. In this approach, half of an electron is promoted from the donor MO to the acceptor MO and the difference in orbital energies at convergence is taken as the electronic transition energy. Due to the necessity of carrying out DFT calculations on Co^{2+}Cbl (an open-shell d^7 system) in a spin-unrestricted manner, the spin-up and spin-down electrons are allowed to occupy spatially and energetically different MOs, and transitions within each set of orbitals thus need to be considered. However, as a result of differences in Coulomb repulsion, the energies obtained for transitions within the spin-down manifold were typically lower than those predicted for their spin-up counterparts. As such, the following discussion is limited primarily to the former set of electronic transitions.

As we were most interested in corroborating our qualitative band assignments with more quantitative methods, the energies of representative transitions belonging to each of the three major classes (LF, MLCT, and $\pi\rightarrow\pi^*$) were calculated using the Slater half-electron excitation methodology. Table 1 provides a list of the electronic transitions considered and their calculated energies. The relevant portion of the spin-down MO diagram computed for Co^{2+}Cbl is presented in Figure 4. The two lowest energy transitions are predicted to involve the excitation of an electron from the Co $3d_{xz}$ and $3d_{yz}$ orbitals to the Co $3d_{z^2}$ orbital

(40) Brooks, A. J.; Fox, C. C.; Marsh, E. N. G.; Vlasie, M.; Banerjee, R.; Brunold, T. C. *Biochemistry* **2005**, in press.
(41) $C_0/D_0 = (kT/\beta H)(\Delta\epsilon_{MCD}/\epsilon_{Abs})$; see ref 14.

Table 1. DFT Computed Energies for Representative Ligand Field (LF), Metal-to-Ligand Charge Transfer (MLCT), and Corrin $\pi\rightarrow\pi^*$ ($\pi\rightarrow\pi^*$) Transitions Obtained Using the Slater Half-Electron Excitation Method within the Spin-Down Electron Manifold[a]

spin-down transition	donor MO	acceptor MO	energy (cm^{-1})	assignment
79 → 80	Co $3d_{xz}$	Co $3d_{z^2}$	9 622	LF1
78 → 80	Co $3d_{yz}$	Co $3d_{z^2}$	11 227	LF2
79 → 81	Co $3d_{xz}$	corrin π^*	15 308	MLCT
77 → 80	Co $3d_{xy}$	Co $3d_{z^2}$	17 381	LF
78 → 81	Co $3d_{yz}$	corrin π^*	17 695	MLCT
77 → 81	Co $3d_{xy}$	corrin π^*	18 978	MLCT
76 → 81	corrin π	corrin π^*	20 212	$\pi\rightarrow\pi^*1$

[a] Isosurface plots of the individual MOs are presented in Figure 4.

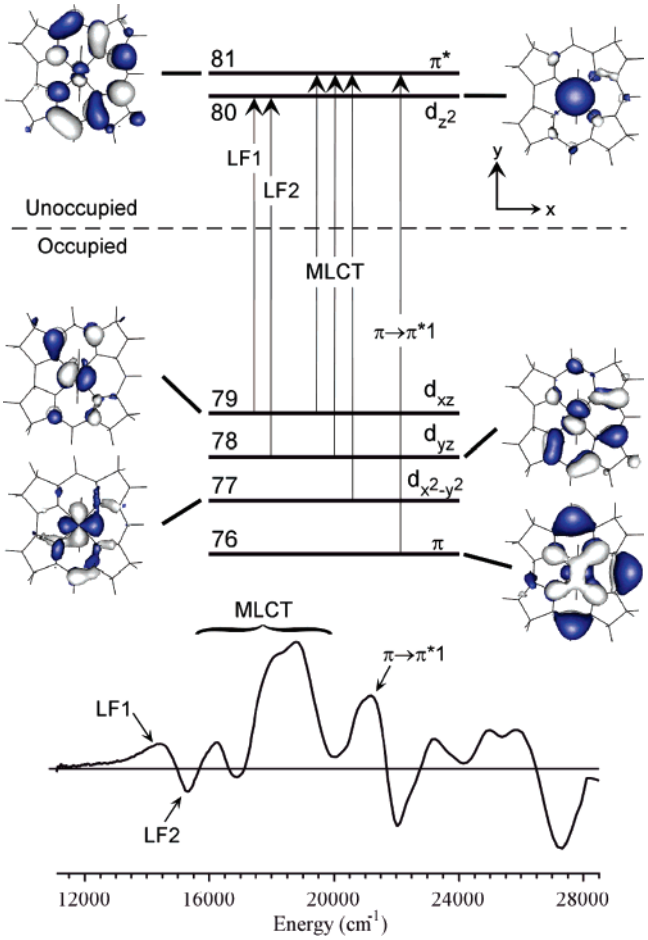

Figure 4. Relative energies and isosurface plots of relevant spin-down molecular orbitals (MOs) for Co^{2+}Cbl based on a spin-unrestricted DFT calculation (top). MOs are designated by their dominant contributors and arranged according to their calculated energies. Electronic transitions that have been correlated to bands in the experimental Co^{2+}Cbl spectrum (bottom) are indicated by arrows in the MO diagram.

(LF1 and LF2, respectively). Although the calculated transition energies for these excitations are lower than the peak position of the lowest-energy band observed in the Co^{2+}Cbl MCD spectrum (Figure 2A), the high C_0/D_0 ratios associated with the first two transitions (Figure 2B) and the absence of bands in the experimental spectrum below 12 000 cm^{-1} suggest that the two lowest-energy MCD features indeed correspond to LF1 and LF2. Additional support for this assignment is provided by EPR data of free Co^{2+}Cbl,[42] as the experimental g-shifts of 0.272 and 0.230 yield estimates for the Co $3d_{xz}\rightarrow 3d_{z^2}$, and Co $3d_{yz}\rightarrow 3d_{z^2}$ transition energies of 11 360 and 13 435 cm^{-1}, respectively, in reasonable agreement with the energies of LF1 and LF2.[13] Our calculations further predict that the three lowest energy spin-down MLCT transitions occur between 15 000 and 19 000 cm^{-1}, in excellent agreement with the general characterization of the Abs and MCD features between 16 000 and 20 500 cm^{-1} as being primarily due to MLCT transitions. However, making specific assignments of the bands across this region is complicated by the prediction of a spin-down LF transition and multiple spin-up MLCT and LF transitions in this same energy range. Nonetheless, it is notable that regardless of their specific origins, a majority of these transitions are calculated to be primarily MLCT in character, in line with the qualitative assignments given above and our previous TD-DFT assignments.[13] The lowest-energy corrin-centered spin-down transition ($\pi\rightarrow\pi^*1$) is predicted at an energy of 20 212 cm^{-1}, in close energetic proximity to the actual peak position of the dominant Abs feature of Co^{2+}Cbl (~21 100 cm^{-1}, Figure 2B) and consistent with the corresponding C_0/D_0 plot (Figure 2B). Although the donor and acceptor MOs involved in the transitions responsible for the remaining intense Abs bands in the near-UV region cannot be identified with certainty, these transitions are likely also primarily $\pi\rightarrow\pi^*$ in nature based on our DFT computations and the experimental C_0/D_0 plot.

Building upon these computationally assisted spectral assignments for Co^{2+}Cbl, changes in the Abs and MCD spectra caused by (i) cofactor binding to the MMCM active site and (ii) the addition of substrate (analogues) to the holoenzyme can now be interpreted in terms of perturbations of the cofactor's electronic structure.

Co^{2+}Cbl/MMCM. The MCD spectrum of MMCM-bound Co^{2+}Cbl is notably different from that of the unbound cofactor (Figure 2A), displaying a relatively uniform blue-shift of the spectral features between 16 000 and 20 000 cm^{-1}, which have been attributed to MLCT transitions. Additionally, Co^{2+}Cbl binding to the MMCM active site gives rise to a general sharpening of the MCD features and, in some cases, an increase in signal intensity (see, for example, the negatively signed component of the derivative-shaped feature centered at 21 600 cm^{-1}). This band sharpening is not unexpected, as the enzyme likely forces the cofactor to adopt a fairly rigid conformation. Most interesting, however, is that the positions of several bands in the Co^{2+}Cbl MCD spectrum are unaffected by cofactor binding to apo-MMCM. These features include the two lowest energy bands attributed to the ligand field transitions LF1 and LF2, respectively (see Figure 4), and the derivative-shaped feature centered at 21 600 cm^{-1}, whose positively signed lower-energy component is attributed to the lowest-energy corrin-centered $\pi\rightarrow\pi^*$ transition ($\pi\rightarrow\pi^*1$ in Figure 4). Considering the overall changes to the MCD spectrum accompanying Co^{2+}-Cbl binding to MMCM, it appears that the enzyme active site is capable of tuning the energy differences between the Co 3d orbitals and the corrin π^* orbitals (as revealed by the blue shift of the MLCT transitions) without dramatically altering the LF splitting and the energies of the corrin π/π^* frontier orbitals (as disclosed by the invariant positions of the LF and corrin $\pi\rightarrow\pi^*$ transitions). The physical origin of this unusual pattern of spectral perturbations is explored in the Discussion section below.

(42) Harmer, J.; Van Doorslaer, S.; Gromov, I.; Schweiger, A. *Chem. Phys. Lett.* **2002**, *358*, 8−16.

Co^{2+}Cbl/MMCM/Substrate (Analogue). As shown in Figure 2A, the addition of substrate (analogues) to the Co^{2+}Cbl/MMCM complex gives rise to further alterations in the MCD spectrum of the cofactor. The region of the spectrum between 17 000 and 20 000 cm^{-1} is most significantly altered upon formation of the substrate (analogue) bound complex and includes the appearance of a unique double-peaked feature centered at 19 500 cm^{-1}. While the exact origin of this feature is unknown, it occurs in the region of the spectrum attributed to MLCT transitions, suggesting that these transitions are significantly perturbed by the addition of substrate (analogues) to MMCM-bound Co^{2+}Cbl. Interestingly, the position of the derivative-shaped feature centered at 21 600 cm^{-1} again does not vary in response to the addition of substrate (analogues), although a significant increase in the intensity of the negatively signed higher-energy component is observed. As the positively signed lower-energy component is assigned as the $\pi\rightarrow\pi^*1$ transition, the relative energies of the corrin π/π^* frontier orbitals of Co^{2+}Cbl also appear largely unaffected by the presence of substrate (analogues) in the enzyme active site.

Discussion

This work represents the first MCD spectroscopic study of a cobalamin-dependent enzyme with Co^{2+}Cbl bound to the active site in the absence and presence of substrate (analogues). Notable differences in the MCD spectra of protein-bound versus free Co^{2+}Cbl are observed that are significantly more pronounced than those reported for MMCM-bound versus free AdoCbl and MeCbl.[6] As such, our MCD spectroscopic studies suggest that perturbation of the Co^{2+}Cbl form, but not the Co^{3+}-Cbl form, of the cofactor is likely a significant source of enzymatic Co−C bond activation.

Intriguingly, our MCD data reveal that, upon formation of the MMCM-bound Co^{2+}Cbl species both in the absence and presence of substrate (analogues), the energies of the MLCT transitions are affected to a significantly greater extent than are those of the LF and corrin-centered $\pi\rightarrow\pi^*$ transitions. We invoke a fairly uniform stabilization of the Co 3d-based MOs serving as donor orbitals in those MLCT transitions (and, because these orbitals are filled, a stabilization of the enzyme-bound cofactor as a whole) to account for these observations, as in this case the energy gap between the metal 3d- and corrin π^*-based MOs would increase without markedly affecting the LF splitting of the Co^{2+} 3d orbitals and the energy separation between the corrin π- and π^*-based MOs.[43]

As the dielectric constants of the medium surrounding the free (in aqueous solution) and MMCM-bound Co^{2+}Cbl cofactor are drastically different, this significant change in solvation could potentially account for the alterations in the electronic transition energies observed experimentally. However, our DFT calculations on the Co^{2+}Cbl cofactor in the presence of a COSMO solvent field with large ($\epsilon = 80$) and small ($\epsilon = 10$) dielectric constants indicate only minor changes in the energies of all calculated transitions below 21 000 cm^{-1} (Table S3, Supporting Information). These results are corroborated by the remarkable similarities between TD-DFT calculated Abs spectra of the Co^{2+}-Cbl cofactor in the presence of a COSMO field with $\epsilon = 80$ and 10 below 21 000 cm^{-1} (Figure S1, Supporting Information). In comparison, parallel calculations carried out for a MeCbl model predict significantly larger spectral changes, particularly in the so-called γ-region between 25 000 and 32 000 cm^{-1} (Figure S2, Supporting Information), and correlate well with our experimental results, where changes to the γ-region are observed upon incorporation of the MeCbl cofactor into the MMCM active site.[6] Thus, while the altered dielectric constant of the protein active site as compared to aqueous solution can account for the spectroscopic changes observed for MeCbl (and AdoCbl), it is insufficient to rationalize those changes reported here for Co^{2+}Cbl.

An alternative, and likely more significant, source of stabilization of the Co 3d orbitals by the protein active site is through reduction of the charge donation from one or several ligands of the cobalt ion. A particularly appealing mechanism by which this reduction in ligand → Co charge donation could occur is through coupling of the Co−C bond homolysis step (Scheme 1) to proton uptake by the "catalytic triad" involved in ligation of the lower face of the Cbl cofactor.[44] Many Cbl-dependent enzymes are known to possess a conserved set of three residues with an established hydrogen bonding network, known as the "catalytic triad"; in MMCM this triad includes His^{610}, Asp^{608}, and Lys^{604}. Proton uptake by this triad in response to the Co^{3+} to Co^{2+} conversion would be expected to reduce the donor strength of the axial His ligand, thereby facilitating formation of enzyme-bound Co^{2+}Cbl by increasing the effective nuclear charge of the central Co atom and, consequently, stabilizing the occupied Co^{2+} 3d orbitals. Coupling of H^+ uptake to metal ion reduction so as to fine-tune reactivity is a strategy employed by a wide variety of redox-active metalloenzymes.[45−47] It should be noted that in the case of MMCM, a weakening of the Co−N_{ax} bonding interaction alone should stabilize the singly occupied Co^{2+} $3d_{z^2}$-derived MO relative to the other metal 3d-based MOs, giving rise to a decrease in the energies of all LF transitions terminating in the former orbital. Experimentally, however, we observe only minor shifts of these transitions (LF1 and LF2, Figure 4) upon Co^{2+}Cbl binding to MMCM. Consequently, our spectroscopic data indicate that additional perturbations to the cofactor must occur in the enzyme active site, such as displacement of the Co^{2+} ion out of the plane defined by the four pyrrolic nitrogen atoms and toward the His^{610} ligand. Such a scenario is intriguing, as it would preserve the LF splitting of the Co^{2+} 3d orbitals while simultaneously also reducing the charge donation from the corrin macrocycle to the cobalt center.

While the data in Figure 2A indicate that the enzyme alone modulates the energies of the Co^{2+} 3d orbitals, the fact that the addition of substrate (analogues) induces additional band shifts implies that stabilization of MMCM-bound Co^{2+}Cbl, and hence the extent of Co−C bond activation for the precursor AdoCbl, is greatest when substrate is present in the active site (probably by placing additional steric constraints on the bound cofactor). This observation suggests that the enzyme has evolved so as to prevent formation of the Co^{2+}Cbl/Ado$^\bullet$ radical pair in the

(43) While the spectroscopic results obtained in this study conclusively demonstrate that the post-homolysis product Co^{2+}Cbl is significantly perturbed by the MMCM active site, no simple correlation exists between the observed MCD band shifts and the extent of enzymatic stabilization of the bound cofactor.

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absence of substrate, thereby protecting itself from deleterious side reactions that could otherwise occur. Additional studies with MMCM mutants and cofactor analogues will help elucidate the role of specific enzymatic interactions in stabilizing Co^{2+}-Cbl.

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Supporting Information Available: Cartesian coordinates for Co^{2+}Cbl and MeCbl computational models, DFT calculated electronic transition energies for Co^{2+}Cbl in the presence of a COSMO solvation field with $\epsilon = 80$ and 10, and TD-DFT calculated Abs spectra and difference spectra for Co^{2+}Cbl and MeCbl in the presence of a COSMO field ($\epsilon = 80$ and 10). This material is available free of charge via the Internet at http://pubs.acs.org.

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