The Coenzyme B<sub>12</sub> Analog 5'-Deoxyadenosylcobinamide-GDP Supports Catalysis by Methylmalonyl-CoA Mutase in the Absence of Trans-ligand Coordination*

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Methylmalonyl-CoA mutase is an 5'-adenosylobalamin (AdoCbl)-dependent enzyme that catalyzes the rearrangement of methylmalonyl-CoA to succinyl-CoA. The crystal structure of this protein revealed that binding of the cofactor is accompanied by a significant conformational change in which dimethylbenzimidazole, the lower axial ligand to cobalt, is replaced by histidine coordination. The role of the lower axial ligand in the trillion-fold labilization of the upper axial cobalt–carbon bond has been the subject of enduring debate in the model inorganic literature. In this study, we have used a cofactor analog, 5'-deoxyadenosylcobinamide GDP (AdoCbi-GDP), which reconstitutes the enzyme in a "histidine-off" form and which allows us to evaluate the contribution of the lower axial ligand to catalysis. The k<sub>cat</sub> for the enzyme in the presence of AdoCbi-GDP is reduced by a factor of 4 compared with the native cofactor AdoCbl. The overall deuterium isotope effect in the presence of AdoCbi-GDP (k<sup>D</sup>/k<sup>H</sup> = 8.6 ± 0.8) is comparable with that observed in the presence of AdoCbl (5.0 ± 0.6) and indicates that the hydrogen transfer steps in this reaction are not significantly affected by the change in coordination state of the bound cofactor. These surprising results are in marked contrast to the effects ascribed to the corresponding lower axial histidine ligands in the cobalamin-dependent enzymes glutamate mutase and methionine synthase.

Coenzyme B<sub>12</sub>, or AdoCbl<sup>1</sup>-dependent enzymes catalyze a wide variety of isomerization reactions in which a migrating group and a hydrogen atom on vicinal carbons exchange positions. A common function of the cofactor in these reactions is to serve as a dormant source of radicals that is activated on substrate binding by homolysis of the organometallic Co–C bond (1–3). The uncatalyzed rate for the cleavage of the Co–C bond in the cofactor free in solution is 3.8 × 10<sup>-19</sup>s at 37 °C (4). In contrast, the k<sub>cat</sub> for most AdoCbl-dependent enzymes is on the order of ~10<sup>23</sup>s, leading to a predicted rate enhancement that is on the order of 1 trillion-fold (5). A member of this class of enzymes is methylmalonyl-CoA mutase, which catalyzes the 1-2 rearrangement of methylmalonyl-CoA to succinyl-CoA (for review, see Refs. 6, 7). It is distinguished by being the only family member that is found in both bacterial and mammalian organisms. Methylmalonyl-CoA mutase catalyzes a 0.9 × 10<sup>12</sup>-fold enhancement of the homolysis rate that corresponds to a lowering of the activation barrier by 17 kcal/mol at 37 °C (8).

In solution and at physiological pH, AdoCbl is six-coordinate, and the lower axial ligand is the bulky and weakly basic intramolecular base dimethylbenzimidazole (9). The potential role of the lower axial ligand in labilizing the upper axial Co–C bond has been the focus of much discussion. A popular hypothesis to explain the observed rate enhancement invokes the role of conformational distortion of the corrin macrocycle (e.g. Refs. 10–14). According to the "mechanochemical" mechanism for labilization of the Co-C bond, an upward flexing of the corrin ring would lead to steric crowding on the β face, thereby weakening the organometalllic bond. The influence of trans-steric and electronic effects exerted by the lower axial ligand has been examined in a number of model compounds (15–19).

On the basis of these model studies, a bulky and weakly basic ligand such as dimethylbenzimidazole would appear to be well suited for AdoCbl-dependent enzymes capable of effecting spectacular enhancements of the Co–C bond homolysis rate. It therefore came as a surprise when spectroscopic investigations (20, 21) and crystal structures (22, 23) revealed that the cofactor was bound in a "dimethylbenzimidazole-off" conformation in some AdoCbl-dependent enzymes. This ligand switch was first reported in B<sub>12</sub>-dependent methyltransferases that catalyze heterolytic cleavage of the Co–C bond (24, 25). In all these enzymes, a histidine residue donated by the protein replaces the intramolecular ligand, dimethylbenzimidazole.

In methylmalonyl-CoA mutase, His<sup>610</sup> is coordinated to the cobalt and is part of a hydrogen-bonding triad involving Asp<sup>608</sup> and Lys<sup>605</sup> (22). We have previously shown that a major role for the dimethylbenzimidazole-containing tail in methylmalonyl-CoA mutase is to organize the active site for catalysis (26). Thus, the cofactor analogs AdoCbi and 5'-deoxyadenosylcobinamide phosphatc methylester, lacking the dimethylbenzimidazole moiety, bind tightly to the enzyme but do not support catalysis. On the basis of comparisons of the crystal structures of holomethylmalonyl-CoA mutase (22) and of the B<sub>12</sub>-binding domain of glutamate mutase lacking the cofactor (27), we had postulated that the role of the bulky base was to organize a disordered loop in the apoenzyme to a helix found in the holoenzyme (26). In this study, we have used an AdoCbi analog, AdoCbi-GDP (Fig. 1), in which the terminal base on the nucle-
otide tail, dimethylbenzimidazole, is replaced by GDP. This derivative supports catalysis with only slightly reduced efficiency but, surprisingly, does so from a "histidine-off" conformation. These results suggest that the lower axial ligand His110
clearly plays a minor role in the reaction catalyzed by methylmalonyl-CoA mutase.

**EXPERIMENTAL PROCEDURES**

**Materials**—AdoCbl, GDP, and methylmalonyl-CoA were purchased from Sigma. Radioactive ([3H]CH3-malonay-CoA (56.4 Ci/mol) was purchased from New England Nuclear. Thio kinase was purchased from Boehringer Mannheim Biochemicals. [CD2]Methylmalonyl-CoA (28) and AdoCbi-GDP (29) were synthesized as described previously. Titanium citrate was prepared as described previously (30). All other chemicals were reagent-grade commercial products and were used without further purification.

**Enzyme Expression and Purification**—The recombinant expression vector pMEX2/GP1-2 harboring the Propionibacterium shermanii genes in Escherichia coli strain K38 (31) was a gift from Peter Leadlay (Cambridge University). The enzyme was purified through the step preceding reconstitution with cofactor as described before (32).

**Enzyme Assays**—The specific activity of the mutase was determined in the radiolabeled assay at 37 °C as described previously (33) and was 26 units/mg of protein. 1 unit of activity catalyzes the formation of 1 μmol of succinyl-CoA/min at 37 °C. The deuterium isotope effect was measured with (CD2)methylmalonyl-CoA in the coupled thio kinase assay at 30 °C as described previously (33).

**Determination of Equilibrium Binding Constants for AdoCbl and AdoCbi-GDP by Fluorescence Spectroscopy**—The intrinsic tryptophan fluorescence emission of methylmalonyl-CoA mutase (monitored at 340 nm) is quenched on binding of AdoCbi-GDP as also seen with AdoCbl fluorescence emission. Free AdoCbi-GDP, even at millimolar concentrations, does not exhibit fluorescence emission between 300 and 600 nm on excitation at 282 nm. As an additional control for nonspecific quenching, the fluorescence of lysozyme was monitored on addition of millimolar AdoCbl-GDP—AdoCbi-GDP by Fluorescence Spectroscopy—AdoCbl-GDP binding data were analyzed as described previously (26). The excitation wavelength was 282 nm (slit width, 3 μm), and emission was observed between 300 and 380 nm (slit width, 3 μm). To determine the equilibrium dissociation constant for AdoCbl-GDP, 500 μl of 0.25 μM methylmalonyl-CoA mutase in 50 mM potassium phosphate buffer, pH 7.5, was used. Successive aliquots (2–5 μl) of a 0.1 mM stock AdoCbl-GDP solution prepared in the same buffer were added to the enzyme solution. After each addition, the mixture was incubated at 4 °C for 30 min before measurement of the fluorescence emission. Free AdoCbl-GDP, even at millimolar concentrations, does not exhibit fluorescence emission between 300 and 600 nm on excitation at 282 nm. As an additional control for nonspecific quenching, the fluorescence of lysozyme was monitored on addition of millimolar concentrations of AdoCbl-GDP. No change in the fluorescence emission was observed. Binding data were analyzed as described previously to obtain the respective Kd values for AdoCbl and AdoCbl-GDP (26).

**Equilibrium Binding Constants Measured by UV-Visible Absorption Spectroscopy**—Binding of AdoCbl and AdoCbi-GDP to methylmalonyl-CoA mutase was followed spectrophotometrically using a Cary-118 spectrophotometer (Olis Instruments), in which the cuvette holder was maintained at 4 °C by a thermostatted water circulator. Methylmалonyl-CoA mutase was followed spectrophotometrically using a Cary-118 spectrophotometer (Olis Instruments), in which the cuvette holder was maintained at 4 °C by a thermostatted water circulator. Methylmалonyl-CoA mutase was followed spectrophotometrically using a Cary-118 spectrophotometer (Olis Instruments), in which the cuvette holder was maintained at 4 °C by a thermostatted water circulator.

**RESULTS**

**Spectral Properties of Methylmalonyl-CoA Mutase Reconstituted with AdoCbl-GDP**—AdoCbl-GDP is an intermediate in the biosynthesis of AdoCbl (34) and exists in the “base-off”2 conformation in solution (Fig. 1). Binding of AdoCbl-GDP to wild-type mutase results in an increase in absorption across the entire spectral range (Fig. 2), as has been observed previously for AdoCbl-GDP (26). The spectrum of the bound cofactor retains the 460-nm absorption maximum, indicating that His110 in the active site is not coordinated to the cobalt in AdoCbl-GDP-reconstituted enzyme (Fig. 1). The increase in absorbance at 460 nm accompanying cofactor binding was plotted as a function of the concentration of free cofactor at equilibrium (Fig. 2, inset). The free ligand concentration was calculated at each point in the titration curve by using the Kd obtained from the fluorescence experiments (4.9 μM) as described below (26). The excellent fit to the absorbance data using the Kd value obtained from the fluorescence data confirms that the binding isotherm obtained by fluorescence spectroscopy reflects specific binding of the cofactor to the mutase.

**Steady-state Kinetic Properties of AdoCbi-GDP-Reconstituted Methylmalonyl-CoA Mutase**—Despite the lower affinity of methylmalonyl-CoA mutase for AdoCbl-GDP, it is a relatively...
good cofactor analog supporting turnover with a $k_{\text{cat}}$ that is only 4-fold lower than the natural cofactor AdoCbl (Table I). The $K_m$ for the substrate methylmalonyl-CoA is not affected by the cofactor analog (data not shown). The overall deuterium isotope effect under steady-state conditions in the presence of AdoCbl ($|\Delta V| = 5.0 \pm 0.6$) and AdoCbi-GDP ($|\Delta V| = 7.2 \pm 0.8$) are similar to each other and to the isotope effect ($|\Delta V| = 6.2$) reported previously for wild-type methylmalonyl-CoA mutase in the presence AdoCbl (35).

**Determination of Equilibrium Binding Constants for Cofactors by Fluorescence Spectroscopy**—Addition of AdoCbl and AdoCbi-GDP to methylmalonyl-CoA mutase results in a decrease in fluorescence emission at 340 nm (data not shown) that can be used to determine the equilibrium dissociation constant (26). The $K_f$ for AdoCbi-GDP is $4.9 \pm 0.3 \mu M$, which is 30-fold higher than that for AdoCbl (Table I).

**Electronic Absorption Spectrum of AdoCbi-GDP-reconstituted Methylmalonyl-CoA Mutase under Steady-state Turnover Conditions**—The spectrum of the enzyme monitored under steady-state turnover conditions reveals the predominance of the AdoCbi-GDP state (Fig. 3). However, the spectrum of the enzyme in the presence of substrate is slightly blue shifted, and the absorption maximum shifts from 460 to 456 nm. Two isosbestic crossovers are observed at 454 and 456 nm. In the presence of the natural cofactor, the steady-state spectrum of the enzyme comprises of a mixture of AdoCbl ($\sim 80\%$) and cob(II)alamin ($\sim 20\%$; Ref. 36).

**EPR Spectrum of Cob(II)iminamide-GDP-reconstituted Methylmalonyl-CoA Mutase**—To confirm that the mutase-bound cofactor was in the base-off conformation, the EPR spectrum of the photolyzed enzyme was recorded (Fig. 4). The axial spectrum is diagnostic of base-off cob(II)iminamide in which the $g_{||}$ component is split into eight lines centered at $g = 2.002$ because of hyperfine coupling between the unpaired electron and cobalt nucleus ($I = 7/2$). The hyperfine coupling constant is 144 G. The $g_{\perp}$ component is broad and poorly resolved, and the overall spectrum resembles that of the clostridial cornoid iron-sulfur protein that also contains a base-off corrin (37). The absence of histidine ligation to cob(II)iminamide-GDP in the mutase active site is indicated by the following two observations. The hyperfine coupling constant, which is 144 G, is typical of a base-off species and notably higher than that expected for a base-on species, which is of the order of 110 G (38). Second, the high-field signals appear as singlets rather than triplets, indicating the absence of superhyperfine coupling between the unpaired electron and an $I = 1$ nucleus, as has been observed for histidine ligation in methylmalonyl-CoA mutase (20). Together, these data provide convincing evidence for the base-off conformation of AdoCbi-GDP bound to the mutase active site.

**DISCUSSION**

Clearly, cobalt coordination by the bulky intramolecular base, dimethylbenzimidazole, is not essential for catalysis in AdoCbl-dependent isomerases. Thus, in a subfamily of these enzymes including methylmalonyl-CoA mutase and glutamate mutase, the nucleotide loop is bound in an extended conformation and is replaced by a histidine ligand donated by the protein (22, 23). In contrast, coordination by dimethylbenzimidazole is preserved in the active site in a second subfamily (39–42).

**Table I**

Comparison of steady-state kinetic parameters with AdoCbl versus AdoCbi-GDP

| Cofactor     | $K_{\text{AdoCbl}}$ ($\mu M$) | $k_{\text{cat}}$ (at 30°C) ($s^{-1}$) | $\Delta V$ (G) |
|--------------|------------------------------|--------------------------------------|----------------|
| AdoCbl       | 0.17 ± 0.01                  | 73                                    | 5.0 ± 0.6      |
| AdoCbi-GDP   | 4.9 ± 0.3                    | 18                                    | 7.2 ± 0.8      |
However, regardless of the identity of the lower axial ligand, its role in catalysis is poorly understood.

In a previous study, we had reported that deletion of the nucleotide base from the cofactor tail resulted in inactive cofactors that retained tight binding to methylmalonyl-CoA mutase (26). In this study, we have examined the binding and catalytic properties of AdoCbi-GDP. Surprisingly, this analog supports catalysis but binds to the active site in a histidine-off conformation and thus permits evaluation of the contribution of the lower axial ligand to the overall reaction.

AdoCbi-GDP differs from AdoCbl in three important structural respects (Fig. 1). First, the anomic carbon in GDP is in the β configuration (versus α in the dimethylbenzimidazole nucleotide of AdoCbl). Second, the nucleotide loop has a diphosphate moiety in AdoCbi-GDP and a monophosphate moiety in AdoCbl. Third, the phosphate group is at the 5′ position of the ribose ring in AdoCbi-GDP and at the 3′ position in AdoCbl. When bound to the mutase, AdoCbi-GDP remains in the base-off conformation, as evidenced by the 460-nm absorption maximum (Fig. 2) and the EPR spectrum (Fig. 4). Thus, it appears that although the occupancy of the nucleotide binding pocket is essential for catalysis, the structure of the nucleotide is not as critical. Consistent with this conclusion is the report that deoxyadenosine-(p-cresol)ecobamide, in which dimethylbenzimidazole is replaced by p-cresol, supports methylmalonyl-CoA mutase activity (43). However, because the spectrum of the enzyme in the presence of this analog was not reported, it is not known whether His610 is coordinated to the cobalt in the active site when p-cresol rather than dimethylbenzimidazole is positioned in the nucleotide pocket.

AdoCbi-GDP binds to methylmalonyl-CoA mutase with an ~30-fold lower affinity (Table I). As a cofactor, AdoCbi-GDP is rather efficient, and the kcat for methylmalonyl-CoA mutase is only 4-fold lower than with AdoCbl. In addition, the overall deuterium isotope effects on the reactions supported by AdoCbl and AdoCbi-GDP are similar (Table I). Thus, the intrinsic deuterium isotope effect associated with hydrogen transfer from deoxyadenosine to substrate (36, 44) is suppressed to a similar extent in both cases. This is significant, because the hydrogen transfer step from substrate to cofactor (and presumably from cofactor to product) is kinetically coupled to the carbocarbon bond homolysis step (36). These results suggest that the absence of histidine coordination does not affect the coupled homolysis step, because the overall energetics of the reaction, as indicated by the isotope effects, are similar.

Thus the lower axial ligand His610 apparently plays a minor role in the reaction catalyzed by methylmalonyl-CoA mutase, contributing marginally, if at all, to the 0.9 × 1012-fold acceleration of the Co–C homolysis reaction rate (8). This is significantly lower than the 870-fold enhancement of the homolysis rate constant attributed to N-methylimidazole coordination to AdoCbi in model studies (45). This could be explained by significant differences in the free energy profiles of the catalyzed and uncatalyzed reactions (8). The seemingly minor role played by the lower ligand in the reaction catalyzed by methylmalonyl-CoA mutase is in apparent contrast to the rather significant changes that have been reported in the related isomerase glutamate mutase (46) and in the methylocobalamin-dependent enzyme methionine synthase (47, 48). In all three enzymes, the DXHXXG motif is seen (49), in which the conserved histidine and aspartate residues are involved in a hydrogen bonding network (“the catalytic triad”; Ref. 25), and the histidine serves as the lower ligand to cobalt. In glutamate mutase, both conservative and nonconservative mutations of the coordinating histidine residue lead to significantly increased Kd values for the cofactor and are accompanied by an ~105-fold lower kcat. In contrast to methylmalonyl-CoA mutase, glutamate mutase binds AdoCbl relatively weakly (Kd = 1.8 μM; Ref. 46). Therefore, coordination by the lower axial ligand may be more important for cofactor binding in glutamate mutase than in methylmalonyl-CoA mutase.

In methionine synthase, mutation of the coordinating histidine residue His759 to glycine results in retention of strong cofactor binding and an ~105-fold diminution in catalytic activity (47). The base-off state in this enzyme is associated with switching the protein conformation from the catalytic to the reductive activation mode (50). Thus, it is likely that the large catalytic penalty incurred by the H759G mutation in methionine synthase resulted from the protein being frozen in an inactive conformation rather than from effects on the chemical steps in the cobalamin-dependent methyltransfer reaction. This is supported by the observation that the susceptibility of the H759G mutant to limited proteolysis is similar to that of inactive wild-type enzyme, suggesting that the enzyme exists in the conformation that binds the repair protein, flavodoxin (51). The effects of conservative mutagenesis at His759 have not been reported.

In summary, our studies reveal that although the absence of dimethylbenzimidazole from its binding pocket is marked by failure of the cofactor to support catalysis and of His610 to coordinate to cobalt, the presence of an analog such as GDP results in a relatively high level of enzyme activity, albeit in the absence of His610 ligation. An alternative and perhaps more direct evaluation of the contribution of His610 to catalysis would be by site-directed mutagenesis of the residue, and these studies are in progress in our laboratory. The present study reveals that the lower axial ligand apparently plays a minor role in the rearrangement reaction catalyzed by methylmalonyl-CoA mutase, and its presence does not confer a significant catalytic advantage. We speculate that retention of the cofactor binding mode with the dimethylbenzimidazole to histidine ligand switch represents an evolutionary vestige in methylmalonyl-CoA mutase, because histidine ligation is not critical for cofactor binding or for the catalytic efficiency of the enzyme.

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