THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 21, Issue of May 24, pp. 18523–18527, 2002
Printed in U.S.A.

Importance of the Histidine Ligand to Coenzyme B_{12} in the Reaction Catalyzed by Methylmalonyl-CoA Mutase*

Received for publication, December 11, 2001, and in revised form, March 7, 2002
Published, JBC Papers in Press, March 13, 2002, DOI 10.1074/jbc.M111809200

Monica Vlasie, Shantanu Chowdhury, and Ruma Banerjee‡

From the Department of Biochemistry, University of Nebraska, Lincoln, Nebraska 68588-0664

Methylmalonyl-CoA mutase is an adenosylcobalamin (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 the cobalt in solution, is replaced by His-610 donated by the active site. The contribution of the lower axial base to the $\sim 10^{12}$-fold rate acceleration of the homolytic cleavage of the upper axial cobalt-carbon bond has been the subject of intense scrutiny in the model inorganic literature. In contrast, *trans* ligand effects in methylmalonyl-CoA mutase and indeed the significance of the ligand replacement are poorly understood. In this study, we have used site-directed mutagenesis to create the H610A and H610N variants of methylmalonyl-CoA mutase and report that both mutations exhibit both diminished activity (5,000- and 40,000-fold, respectively) and profoundly weakened affinity for the native cofactor, AdoCbl. In contrast, binding of the truncated cofactor analog, adenosylcobinamide, lacking the nucleotide tail, is less impaired. The catalytic failure of the His-610 mutants is in marked contrast to the phenotype of the adenosylcobinamide-GDP reconstituted wild type enzyme that exhibits only a 4-fold decrease in activity, although His-610 fails to coordinate when this cofactor analog is bound. Together, these studies suggest that His-610 may: (i) play a structural role in organizing a high affinity cofactor binding site possibly via electrostatic interactions with Asp-608 and Lys-604, as suggested by the crystal structure and (ii) play a role in catalyzing the displacement of dimethylbenzimidazole thereby facilitating the conformational change that must precede cofactor docking to the mutase active site.

Coenzyme B_{12} or AdoCbl[1]-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 by homolysis of the organometallic Co–C bond upon substrate binding. The uncatalyzed rate for the cleavage of the Co–C bond in the cofactor free in solution is 3.8×10^{-9} s^{-1} at 37 °C (1). In contrast, the k_{cat} for most AdoCbl-dependent enzymes is of the order of $\sim 10^2$ s^{-1} leading to a predicted rate enhancement that is of the order of 10^{12}-fold (2). A member of this class of enzymes is methylmalonyl-CoA mutase, which catalyzes the 1,2 rearrangement of methylmalonyl-CoA to succinyl-CoA (reviewed in Ref. 3). 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^{12}-fold enhancement of the homolysis rate that corresponds to a lowering of the activation barrier by 17 kcal/mol at 37 °C (4).

Unlike the porphyrins and chlorins, other members of the family of tetrapyrrolic-derived cofactors, cobalamins are characterized by the presence of a large peripheral ornamentation appended from ring D, the dimethylbenzimidazole-containing nucleotide loop. In solution and at physiological pH, AdoCbl is six-coordinate, and the lower axial ligand is the bulky and weakly basic intramolecular base, dimethylbenzimidazole (5). However, in the class I or "His-on" (3) subfamily of AdoCbl-dependent enzymes, this lower ligand is replaced by a histidine residue provided by the respective proteins (6, 7), while the dimethylbenzimidazole moiety is held >10 Å away from the cobalt. This ligand switch was first reported in B_{12}-dependent methyltransferases that catalyze heterolytic cleavage of the Co–C bond (8, 9). In contrast, the class II or "dmb-on" enzymes retain the intramolecular ligand in their bound conformation (10–12).

The potential role of the lower axial ligand in labilizing the upper axial Co–C bond has been the focus of enduring debate. A popular hypothesis to explain the observed rate enhancement invokes the role of conformational distortion of the corrin macrocycle (for example, see Refs. 13–18). According to this "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 organometallic bond.

The influence of the *trans* electronic effects on the cobalt-alkyl bond dissociation energies have been determined with phenylethylcobalt dimethylglyoxime model compounds in which in a series of substituted pyridines were employed as the *trans* ligand (16). These studies revealed a linear correlation between increasing basicity of the *trans* ligand and increasing stabilization of the Co–C bond, consistent with the formal reduction of Co^{3+} to Co^{2+} during homolysis. *Trans* steric effects were evaluated in a series of benzylcobalt dimethylglyoxime model complexes in which the bulk of the tertiary phosphine ligand was varied. An inverse correlation between size and Co–C bond dissociation energy was noted (19). These results were consistent with studies on Co-dimethylglyoxime complexes in which increasing the size of the *trans* ligand resulted in a corresponding lengthening of the Co–C bond (20, 21). However, a study on the association of a series of substituted

* This work was supported by Grant DK45776 from the National Institutes of Health (to R. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ An Established Investigator of the American Heart Association. To whom correspondence should be addressed. Tel.: 402-472-2941; Fax: 402-472-7842; E-mail: rbanerjee1@unl.edu.

[1] The abbreviations used are: AdoCbl, 5′-deoxyadenosylcobalamin or coenzyme B_{12}; dAdo, 5′-deoxyadenosine; AdoCbi, 5′-deoxyadenosylcobinamide; AdoCbi-GDP, 5′-deoxyadenosylcobinamide guanosine diphosphate; *N*-MeIm, *N*-methylimidazole; dmb, dimethylbenzimidazole.

phosphines to AdoCbi failed to obtain any evidence for binding, indicating that alkylcobaloximes are probably poor models for the biologically relevant corrinoid cofactors (22).

Sirovatka and Finke have compared the thermolysis of AdoCbi in the presence and absence of *N*-MeIm as a mimic of the His-on class of isomerases (23). The key finding from these solution studies is that *N*-MeIm significantly reduces the proportion of Co–C bond homolysis from ≥98% in AdoCbl to ~50% in AdoCbi·*N*-MeIm, with the remaining cleavage occurring via the heterolytic pathway, and it accelerates Co–C bond homolysis and heterolysis by a factor of 8 and 350, respectively, relative to AdoCbl. In addition, the presence of *N*-MeIm leads to an 870-fold and 3×10^4–fold increase in the homolytic and heterolytic rates, respectively, for Co–C bond cleavage relative to AdoCbi in the solvent, ethylene glycol.

To address the role of imidazole ligation in a biological context, we have examined the effect of mutating His-610, the residue provided by the *Propionibacterium shermanni* methylmalonyl-CoA mutase. In this enzyme, His-610 is coordinated to the cobalt and is part of a hydrogen-bonding triad involving Asp-608 and Lys-604 (Fig. 1 and Ref. 6). Our study indicates that binding of AdoCbl is more severely affected than binding of AdoCbi, the cofactor lacking the dimethylbenzimidazole ribose phosphate moiety. We propose that the low activity of the H610A and H610N mutants of methylmalonyl-CoA mutase results from weak binding of the cofactor, and that a major role of the histidine may be in dimethylbenzimidazole displacement and in organization of the active site for tight cofactor binding.

EXPERIMENTAL PROCEDURES

Materials—AdoCbl and methylmalonlyl-CoA were purchased from Sigma. Radioactive [^{14}C]CH_3-malonyl-CoA (56.4 Ci/mol) was purchased from PerkinElmer Life Sciences. All other chemicals were reagent grade commercial products and were used without further purification. AdoCbi was synthesized as described previously (24) and characterized by FAB-MS analysis at the Midwest Center for Mass Spectroscopy at the University of Nebraska, Lincoln.

Construction of Site-specific Mutants—A 451-bp *Eco*81I-*Hin*dIII fragment from pMEX2 containing the *P. shermanii* methylmalonyl-CoA mutase gene (Ref. 25, provided by P. Leadlay at the University of Cambridge) was subcloned into the pBS vector (Stratagene), which was then used as a template for creation of mutations by using the strategy supplied with the QuikChange kit (Stratagene). The following sense mutagenic primers were employed for PCR: H610A, GCCAGGACGGTGCCGACCG**C**GGCCAGAAGGTC and H610N, GCCAGGACGGTAACGACCG**C**GGCCAGAAGGTC. The mutant codons are underlined in each case. Each antisense mutagenic primer had the respective complementary sequence and boundaries that were identical to that of the sense primers. In each case, a *Sac*II restriction site was introduced by changing a T to a C (bold) without changing the identity of the encoded amino acid (Arg), to facilitate selection of transformants containing the mutant sequences. T3 and T7 primers were used to confirm the presence of the mutation in the pBS plasmid by nucleotide sequence determination.

The mutation-bearing insert was excised from pBS by restriction digestion with *Eco*81I and *Hin*dIII and partial *Pst*I restriction digestion and was ligated to the parent expression plasmid, pMEX2, digested with the same restriction enzymes. The presence of the mutation was confirmed by nucleotide sequence determination of both strands as well as by the introduction of a new *Sac*II site at position 4,112 in the pMEX2 plasmid. DNA sequencing was performed at the Biotechnology Center Core facility at the University of Nebraska, Lincoln.

Enzyme Expression and Purification—The mutant enzymes were purified through the step preceding reconstitution with cofactor using a modified procedure described previously for the isolation of wild type enzyme (26). Butyl-agarose (Sigma) was used for hydrophobic chromatography instead of phenyl-Sepharose in the second step, and the Matrex gel blue affinity column was replaced by a POROS anion exchange column (20HQ, Perspective Biosystem) operated on a BIOcad work station. A 10 × 5-cm butyl-agarose column was eluted with a linear gradient ranging from 1.4 to 1 M ammonium sulfate in 50 mM potassium phosphate buffer, pH 7.5, and methylmalonyl-CoA mutase eluted at ~1.2 M ammonium sulfate. The enzyme was concentrated and dialyzed against 50 mM potassium phosphate buffer, pH 7.5, before being loaded onto a POROS 20HQ anion exchange column. The enzyme was eluted with a linear gradient ranging from 0 to 250 mM NaCl in 50 mM potassium phosphate buffer, pH 7.5, at a flow rate of 10 ml min^{-1}. Methylmalonyl-CoA mutase eluted at ~170 mM NaCl. Protein concentration was determined using the Bradford reagent (BioRad) with bovine serum albumin as a standard.

Enzyme Assays—Specific activity of the mutase was determined in the radiolabeled assay at 37 °C as described previously (27). 1 unit of activity catalyzes the formation of 1 μmol of succinyl-CoA min^{-1} at 37 °C. The concentration of mutant enzymes was increased 5,000-fold (with H610N) or 1,000-fold (with H610A) with respect to the wild type enzyme in the standard assay. Kinetic parameters for the two mutants were determined by increasing the duration of the fixed timed assay from 3 to 10 min in the presence of varying concentrations of [^{14}C]-methylmalonyl-CoA (56–2190 μM) or AdoCbl (from 0.14 to 50 μM).

Attempted Determination of Equilibrium Binding Constants for Cofactors by Fluorescence Spectroscopy—Addition of AdoCbl or AdoCbi to methylmalonyl-CoA mutase results in a decrease in fluorescence emission at 340 nm and has been used to determine the equilibrium dissociation constant for the wild type enzyme (28). However, we were unable to use this method with the two His-610 mutants to measure a K_d for AdoCbl or AdoCbi because of the absence of observable changes at low cofactor concentrations and general quenching of protein fluorescence at high concentrations of cofactor. We have attributed this fluorescence quenching at micromolar cofactor concentrations to a filter effect resulting from nonspecific interactions between the cofactor and the protein (28). This property interfered with our ability to use fluorescence spectroscopy to distinguish between binding at the active site *versus* nonspecific binding and suggested that the mutant enzymes display weakened binding affinity.

Equilibrium Binding Constants Measured by UV-Visible Absorption Spectroscopy—Binding of AdoCbi to the mutants 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. Methylmalonyl-CoA mutase (24.5 μM) in 150 μl of 50 mM potassium phosphate buffer, pH 7.5, was employed as a blank. Spectra were recorded between 800 and 306 nm after each addition of AdoCbi (3–5-μl aliquots prepared in the same buffer), following incubation at 4 °C for 30 min. The final AdoCbi concentration used in these experiments was 90 μM. The change in absorbance at 448 nm at each concentration of AdoCbi was obtained by subtracting the spectrum of the same concentration of free AdoCbi in 50 mM potassium phosphate buffer, pH 7.5. The K_d for AdoCbi was determined using Equation 1 as described previously (28).

$$1/(1 - y) = L_o/y(1/K_d) - E_o/K_d \quad \text{(Eq. 1)}$$

Estimation of the binding of AdoCbl to the mutants was attempted using a similar procedure. However, we were able to monitor small absorbance changes at 460 nm only when very high concentrations of AdoCbl (0.2–0.4 mM) were added to 150 μM enzyme. The high background absorption from the free cofactor at these concentrations limited the feasibility of this experiment but provided a lower estimate for the K_d for AdoCbl of ≫300 μM. Absorbance changes could not be detected when low enzyme concentrations (~25 μM) were employed.

RESULTS

Steady State Kinetic Properties of Mutant Proteins—Methylmalonyl-CoA mutases containing the conservative mutation H610N or the nonconservative mutation H610A were purified to near homogeneity using conditions developed for the wild type enzyme. Whereas both mutations had a profound influence on AdoCbl binding (discussed below), they displayed at most a 2–3-fold higher K_m for the cofactor and substrate, respectively (Table I). The K_m for methylmalonyl-CoA for H610N was indistinguishable from the value for the wild type enzyme within the limits of experimental error, whereas the H610A variant had a ~3-fold higher K_m. In both mutants, the $K_{m(app)}$ for AdoCbl was ~2-fold higher. Both mutations resulted in significant decreases in k_{cat}, making these measurements experimentally difficult. Surprisingly, the conservative mutation, H610N, was the poorer catalyst with a k_{cat} of 0.003 s^{-1} at 37 °C *versus* 0.024 s^{-1} for H610A, albeit both were significantly lower than the value of 120 s^{-1} at 37 °C for the wild type enzyme. The

TABLE I
Summary of steady state kinetic parameters for wild type and His-610 mutants of methylmalonyl-CoA mutase

Enzyme	$K_{m(\text{M-CoA})}$	$K_{m(\text{AdoCbl})}$	$K_{d(\text{AdoCbl})}$	$K_{d(\text{AdoCbi})}$	k_{cat} (at 37 °C)
	μM	μM	μM	μM	
Wild type	133 ± 37	0.24 ± 0.02	0.17 ± 0.01	0.62 ± 0.09	120 s^{-1}
H610N	102 ± 8	0.52 ± 0.08	>>300	55.8 ± 5.4	0.003 s^{-1}
H610A	364 ± 42	0.45 ± 0.06	>>300	49.5 ± 2.7	0.024 s^{-1}

low activities of both His-610 mutants raised concerns about possible low level contamination with wild type enzyme. However, the difference in the catalytic activities between the two mutants was consistently observed in every preparation. This would not be expected if low levels of contaminating wild type enzyme were contributing to the observed activities, because the contribution to both mutants should be similar, statistically. In addition, the K_m values for the substrate and cofactor for the mutants was distinct from those of the wild type enzyme (Table I).

Addition of imidazole to the assay mixture up to a concentration of 25 mM did not change the activity of the mutants indicating failure to rescue the functional effects of histidine removal (not shown). In addition, enzyme activity could not be detected when AdoCbl was replaced with AdoCbi in the reaction mixture in the presence or absence of imidazole as observed previously with wild type enzyme (28). In contrast to the wild type enzyme, which displays an overall deuterium isotope effect (^{D}V of 5 ± 0.6), neither the H610A nor the H610N mutant exhibited an isotope effect (data not shown).

Spectral Properties of Methylmalonyl-CoA Mutase Reconstituted with AdoCbl—The weak binding of AdoCbl to the His-610 variants of methylmalonyl-CoA mutase precluded an accurate determination of the K_d for the cofactor. The spectrum of H610A reconstituted with AdoCbl was weak and had features corresponding to a mixture of base-off AdoCbl (460 nm) and hydroxycobalamin (350 and 535 nm) as shown in Fig. 2. The reconstitution procedure used to generate this spectrum is routinely employed with wild type methylmalonyl-CoA mutase and AdoCbl in the laboratory and does not lead to loss of the adenosyl group. These results indicate that the His-610 mutants display enhanced lability of the Co–C bond leading to inactive enzyme containing hydroxycobalamin, a property that is not exhibited by the wild type enzyme. A similar lability has been observed with wild type enzyme reconstituted with AdoCbi, which is in the His-off conformation in the active site (28).

Spectral Properties of Methylmalonyl-CoA Mutase Reconstituted with AdoCbi—Binding of AdoCbi to wild type mutase results in an increase in absorption across the entire spectral range as has been observed previously for AdoCbl (28). In contrast, the difference spectrum of the [His-610]-enzyme·AdoCbi complex obtained following successive additions of cofactor aliquots showed a number of distinct changes (see Fig. 3). These included increases in absorption at 360, 448, 507, and 535 nm and revealed conversion of AdoCbi to hydroxycobinamide in the presence of the enzyme. These results indicate that the absence of the coordinating histidine ligand enhances cleavage of the Co–C bond of AdoCbi as also seen with AdoCbl (see above). The increase in absorbance at 448 nm accompanying cofactor binding was plotted as a function of the concentration of free cofactor and yielded a K_d of 49.5 ± 2.7 μM for H610A (Fig. 3, *inset*) and 55.8 ± 5.4 μM for H610N (Table I).

DISCUSSION

This study was originally designed to evaluate the role of *trans* effects in variants of methylmalonyl-CoA mutase in which the lower, protein-derived ligand, His-610, was either removed (H610A) or altered (H610N). Both changes had profound effects on the cofactor dissociation constant and on k_{cat} but had modest effects on substrate and cofactor K_m values. Thus, the H610A and H610N mutants displayed 5,000- and 40,000-fold diminution in k_{cat}, respectively, as compared with the wild type enzyme, indicating a change in the rate-limiting step that is believed to be product dissociation for the wild type enzyme (29). Our inability to measure the binding constant for AdoCbl by difference UV-visible electronic spectroscopy puts a lower limit on the K_d values for the mutants at ≫300 μM, indicating that the equilibrium dissociation constant has been weakened by ≫1,700-fold.

The marked difference in the apparent K_m and K_d for AdoCbl displayed by the two His-610 mutants is surprising. However, very similar behavior has been reported for the corresponding mutants in glutamate mutase, H16G and H16Q, where the K_m is increased 3–5-fold whereas the K_d is increased an estimated 50-fold (30). As pointed out by Chen and Marsh (30), a minimal kinetic scheme describing the cofactor and substrate binding steps and catalysis reveals that K_m is a complex kinetic term that could in principle be equivalent to, larger, or smaller than K_d. In wild type methylmalonyl-CoA mutase, the K_d and apparent K_m for AdoCbl are equivalent (28, 31). The large difference between the values of the two kinetic constants displayed by the mutants is consistent with a significant change in the binding affinity for AdoCbl, which in turn impacts k_{cat}, presumably by changing the rate-limiting step. This conclusion is supported by suppression of the overall deuterium isotope effect in the mutant enzymes in contrast to that of wild type enzyme that displays a ^{D}V of 5 ± 0.6 (32). This is consistent with a step such as cofactor binding limiting catalysis.

The phenotypes of the His-610 mutants reported in this study are in sharp contrast to the mild effects observed when the His-off state is achieved using the cofactor analog, AdoCbi-GDP (32). The latter is a biosynthetic intermediate in the assembly of cobalamin, and we have previously shown that it supports catalysis by methylmalonyl-CoA mutase. Binding of AdoCbi-GDP results in retention of the 460-nm absorption maximum observed for the cofactor free in solution indicating that His-610 does not move into coordination position when this cofactor analog binds. This is confirmed by the EPR spectrum of bound cob(II)inamide-GDP obtained by photolysis of methylmalonyl-CoA mutase reconstituted with AdoCbi-GDP in which the $A_{\text{Co-N}}$ value for the hyperfine interaction is 144 G, and superhyperfine splittings associated with axial nitrogen ligation are absent. The K_d for AdoCbi-GDP is 4.9 μM *versus* 0.17 μM for AdoCbl, and the k_{cat} is decreased from 73 s^{-1} to 18 s^{-1} at 30 °C. The spectra of the enzyme in the presence and absence of high concentrations of substrate do not show significant changes (32). Because the k_{cat} under maximal velocity conditions is only 4-fold lower with AdoCbi-GDP than with AdoCbl, the lack of observable spectral changes argues against transient coordination by His-610 under catalytic turnover conditions.

Thus, the His-off state achieved by site-directed mutagenesis as in the H610A mutant (and most likely also in the H610N variant), is functionally different from the His-off state ob-

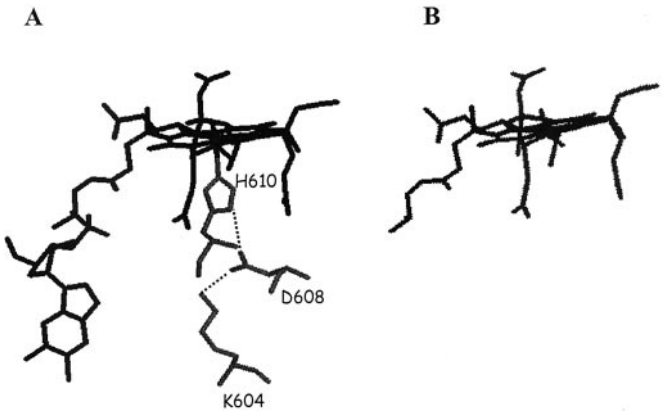

FIG. 1. **Conformations of cobalamin bound to the active site of methylmalonyl-CoA mutase (*A*) and of cobinamide (*B*).** The figures were generated from the protein data bank file, 1REQ1, and the upper axial deoxyadenosine ligand is absent. In *A*, the triad of residues that form a hydrogen-bonding network, Lys-604, Asp-608, and His-610 are shown. When AdoCbi binds to the mutase active site, His-610 is not coordinated to the cobalt.

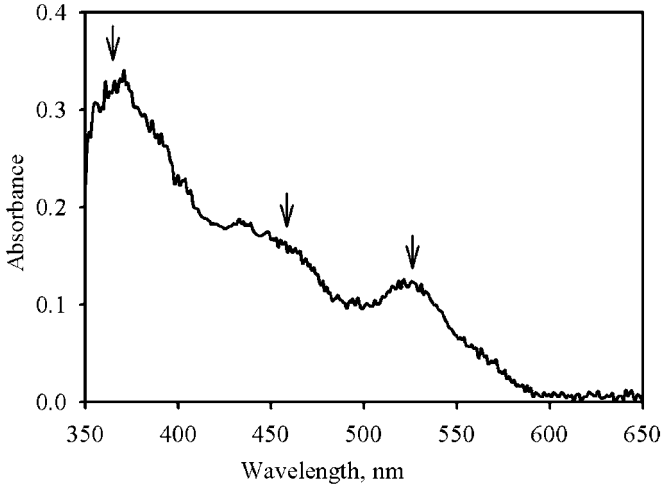

FIG. 2. **Spectrum of H610A reconstituted with AdoCbl.** Enzyme (150 μM) in 50 mM potassium phosphate buffer, pH 7.5, was mixed with 3 mM AdoCbl in the dark and kept on ice for 2 h. Excess cofactor was removed by dialysis against the same buffer. The spectrum obtained has absorption features corresponding to a mixture of hydroxycobalamin (358 and 535 nm) and base-off AdoCbl (460 nm) as indicated by *arrows*.

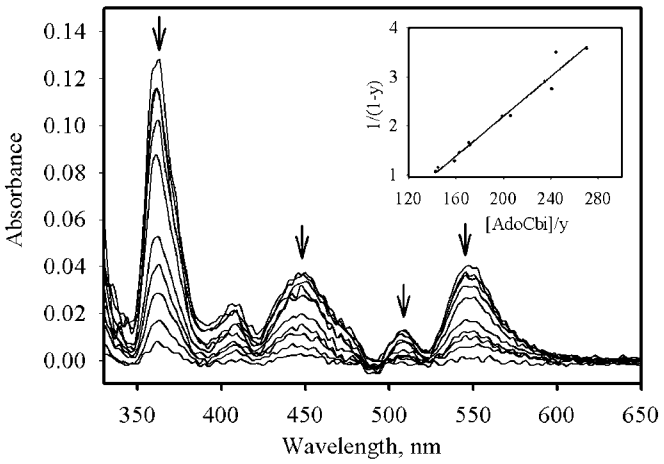

FIG. 3. **Binding of AdoCbi to H610A methylmalonyl-CoA mutase determined by electronic absorption spectroscopy.** The stack plot represents difference UV-visible absorption spectra obtained by successive addition of AdoCbi aliquots to the H610N mutant (24.5 μM) in 50 mM potassium phosphate buffer, pH 7.5. The *arrows* indicate difference absorption maxima at 360, 448, 507, and 535 nm. *Inset*, dependence of absorption change at 448 nm on the concentration of AdoCbi. The data were fit to Equation 1 and yielded a K_d of 49.5 ± 2.7 μM.

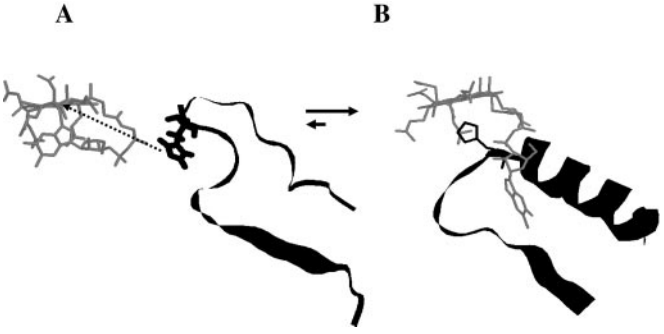

FIG. 4. **Model showing potential role of His-610 in displacement of dimethylbenzimidazole preceding docking of AdoCbl in the mutase active site.** *A*, predocking conformations of cobalamin in solution and residues 1–30 of apoglutamate mutase (PDB: 1BE1) in which His-16, the residue that coordinates to the cofactor is shown in *stick display*. *B*, postdocking conformations of cobalamin and residues 599–625 of methylmalonyl-CoA mutase (PDB: 1REQ1) in which His-610, the residue that coordinates to the cofactor, is shown in *stick display*.

tained with the cofactor analog, AdoCbi-GDP, and raises the question of what the role of His-610 really is. One possibility is that electrostatic interactions between His-610, Asp-608, and Lys-604 may play an important role in catalysis by maintaining a high affinity cofactor binding site (Fig. 1) and that the absence of this hydrogen-bonding network compromises structural integrity in the holoenzyme. This is supported by the observed increase in the rate of Co–C bond cleavage of bound AdoCbl and AdoCbi (Figs. 2 and 3) in comparison to wild type enzyme, where geminate recombination of the homolysis products is greatly favored in the absence of substrate (4).

A second possibility is suggested by the magnitude of the impairment in the equilibrium binding constant when the nucleotide tail is absent as in AdoCbi (~80-fold), which is significantly smaller than when the tail is present as in the natural cofactor, AdoCbl (≫1700-fold, Table I). This suggests that the histidine may be important in binding of the cofactor, specifically in the ligand displacement reaction that must precede docking of AdoCbl to the active site of methylmalonyl-CoA mutase (Fig. 4). The equilibrium association constant for the intramolecular base, dimethylbenzimidazole, in AdoCbl is reported to be 14.3 at 25 °C (33) and the pK_a for this ligand in AdoCbl is 3.7 (34). Thus, at physiological pH, the cofactor exists predominantly in the dmb-on conformation, and the dimethylbenzimidazole must be displaced to achieve the dmb–off conformation in the active site.

We have previously characterized the pH dependence of AdoCbl binding to wild type mutase by stopped-flow fluorescence spectroscopy and found it to be associated with a pK_a of 7.3 (28). In contrast, when the truncated cofactor analog AdoCbi was employed, binding of the cofactor was complete within the instrument dead time, suggesting that dissociation of the dimethylbenzimidazole ligand is the slow step in the docking of the native cofactor to the mutase active site. Based on the results reported here on the His-610 mutants, we propose a model in which the coordinating histidine plays a role in promoting the dmb-off conformation and thereby catalyzes cofactor binding (Fig. 4). The observed pK_a of 7.3 associated with AdoCbl binding would be consistent with ionization of a histidine residue.

Comparison of the highly homologous structures of the B_{12} binding domains of glutamate mutase determined in the ab-

sence of the cofactor (35) and of methylmalonyl-CoA mutase solved in the presence of the cofactor (6), reveals that the domain is largely preorganized and that the ligand histidine in both cases is located on a loop (Fig. 4). The difference, however, is that in the presence of B_{12}, the loop leads to a relatively long α-helix that interacts with the nucleotide tail; however, this stretch of amino acids is largely disordered in the absence of B_{12}. Thus, binding of the cofactor appears to shift the equilibrium toward a more stable α-helix extending from the loop carrying the ligand histidine (36) and presumably helps position it for coordination.

It is noteworthy that the properties of the histidine ligand mutation, H759G, in methylcobalamin-dependent methionine synthase are distinct from those exhibited by the AdoCbl-dependent methylmalonyl-CoA mutase and glutamate mutase with respect to cofactor binding. Tight binding of the cofactor is retained by this mutant although the catalytic efficiency of the enzyme is greatly impaired (37). The ligand histidine in methionine synthase is a member of a histidine-aspartate-serine triad of residues that appears to be important as a proton shunt that facilitates cycling of the cofactor between six-coordinate methylcobalamin and four-coordinate cob(I)alamin states (9). An analogous role is not expected for the isomerases, and indeed, the serine is not conserved in these enzymes. In fact, the significance of the His-on conformation in the subclass of isomerases that exhibit this binding motif is unclear, and it has been speculated that it may represent an evolutionary vestige inherited from the probably more ancient B_{12}-dependent methyltransferase family of enzymes (3). In a second subclass of isomerases, AdoCbl is in fact bound in the dmb-on conformation (11, 12, 38) revealing that catalysis of isomerase chemistry is not uniquely dependent on the presence of the histidine *versus* dimethylbenzimidazole ligand.

Although reconstitution of most B_{12}-dependent enzymes with their respective cofactors is readily achieved *in vitro*, little is known about the process *in vivo*, and in particular, whether or not B_{12} chaperones are involved. Because intermediates in the cofactor biosynthetic pathway have been shown to bind tightly to several B_{12} enzymes, facilitation of this process by proteins would appear to be important for achieving specificity. Depending on the class of B_{12} enzymes, discrimination between the ligand identities at the upper axial position and retention or replacement of the ligand at the lower axial position will be important determinants for cofactor binding.

In summary, characterization of the His-610 mutants of methylmalonyl-CoA mutase in this study in combination with our previous studies on the catalysis of this reaction by AdoCbi-GDP reconstituted enzyme that yields the His-off conformation (32), suggests a role for the histidine residue that has not been considered previously. We propose that the histidine residue may be involved in catalyzing the conformational change from the dmb-on to dmb-off state that must precede AdoCbl docking in the active site and that the primary influence of the histidine residue is on cofactor binding rather than catalysis.

REFERENCES

1. Waddington, M. D., and Finke, R. G. (1993) *J. Am. Chem. Soc.* **115,** 4629–4640
2. Hay, B. P., and Finke, R. G. (1987) *J. Am. Chem. Soc.* **109,** 8012–8018
3. Banerjee, R. (2001) *Biochemistry* **40,** 6191–6198
4. Chowdhury, S., and Banerjee, R. (2000) *Biochemistry* **39,** 7998–8006
5. Lenhert, P. G., and Hodgkin, D. C. (1961) *Nature* **192,** 937–938
6. Mancia, F., Keep, N. H., Nakagawa, A., Leadlay, P. F., McSweeney, S., Rasmussen, B., Bösecke, P., Diat, O., and Evans, P. R. (1996) *Structure* **4,** 339–350
7. Reitzer, R., Gruber, K., Jogl, G., Wagner, U. G., Bothe, H., Buckel, W., and Kratky, C. (1999) *Structure Fold Des.* **7,** 891–902
8. Stupperich, E., Eisinger, H. J., and Albracht, S. P. J. (1990) *Eur. J. Biochem.* **193,** 105–109
9. Drennan, C. L., Huang, S., Drummond, J. T., Matthews, R., and Ludwig, M. L. (1994) *Science* **266,** 1669–1674
10. Yamanishi, M., Yamada, S., Muguruma, H., Murakami, Y., Tobimatsu, T., Ishida, A., Yamauchi, J., and Toraya, T. (1998) *Biochemistry* **37,** 4799–4803
11. Abend, A., Bandarian, V., Nitsche, R., Stupperich, E., Retey, J., and Reed, G. H. (1999) *Arch. Biochem. Biophys.* **370,** 138–141
12. Lawrence, C. C., Gerfen, G. J., Samano, V., Nitsche, R., Robins, M. J., Retey, J., and Stubbe, J. (1999) *J. Biol. Chem.* **274,** 7039–7042
13. Grate, J. H., and Schrauzer, G. N. (1979) *J. Am. Chem. Soc.* **101,** 4601–4611
14. Marzilli, L. G., Toscano, J., Randaccio, L., Bresciani-Pahor, N., and Calligaris, M. (1979) *J. Am. Chem. Soc.* **101,** 6754–6756
15. Chemaly, S. M., and Pratt, J. M. (1980) *J. Chem. Soc. Dalton Trans.* 2274–2281
16. Ng, F. T. T., Rempel, G. L., and Halpern, J. (1982) *J. Am. Chem. Soc.* **104,** 621–623
17. Glusker, J. P. (1982) in *B_{12}* (Dolphin, D., ed), Vol. 1, pp. 23–106, Wiley, New York
18. Brown, K. L., and Brooks, H. B. (1991) *Inorg. Chem.* **30,** 3420–3430
19. Geno, M. K., and Halpern, J. (1987) *J. Am. Chem. Soc.* **109,** 1238–1240
20. Randaccio, L., Bresciani-Pahor, N., Toscano, P. J., and Marzilli, L. G. (1980) *J. Am. Chem. Soc.* **102,** 7373–7375
21. Summers, M. F., Toscano, P. J., Bresciani-Pahor, N., Nardin, G., Randaccio, L., and Marzilli, L. G. (1983) *J. Am. Chem. Soc.* **105,** 6259–6293
22. Garr, C. D., Sirovatka, J. M., and Finke, R. G. (1996) *Inorg. Chem.* **35,** 5912–5922
23. Sirovatka, J. M., and Finke, R. G. (1997) *J. Am. Chem. Soc.* **119,** 3057–3067
24. Ishida, A., and Toraya, T. (1993) *Biochemistry* **32,** 1535–1540
25. McKie, N., Keep, N. H., Patchett, M. L., and Leadlay, P. F. (1990) *Biochem. J* **269,** 293–298
26. Padmakumar, R., and Banerjee, R. (1995) *J. Biol. Chem.* **270,** 9295–9300
27. Taoka, S., Padmakumar, R., Lai, M.-t., Liu, H.-w., and Banerjee, R. (1994) *J. Biol. Chem.* **269**, 31630–31634
28. Chowdhury, S., and Banerjee, R. (1999) *Biochemistry* **38,** 15287–15294
29. Padmakumar, R., Padmakumar, R., and Banerjee, R. (1997) *Biochemistry* **36,** 3713–3718
30. Chen, H. P., and Marsh, E. N. (1997) *Biochemistry* **36,** 7884–7889
31. Maiti, N., Widjaja, L., and Banerjee, R. (1999) *J. Biol. Chem.* **274,** 32733–32737
32. Chowdhury, S., Thomas, M. G., Escalante-Semerena, J. C., and Banerjee, R. (2001) *J. Biol. Chem.* **276,** 1015–1019
33. Hay, B. P., and Finke, R. G. (1984) *Polyhedron* **7,** 1469
34. Brown, K. L., and Hakimi, J. M. (1984) *J. Am. Chem. Soc.* **106,** 7894–7899
35. Tollinger, M., Konrat, R., Hilbert, B. H., Marsh, E. N. G., and Krauetler, B. (1998) *Structure* **6,** 1021–1033
36. Tollinger, M., Eichmuller, C., Konrat, R., Huhta, M. S., Marsh, E. N., and Krautler, B. (2001) *J. Mol. Biol.* **309,** 777–791
37. Jarrett, J. T., Amaratunga, M., Drennan, C. L., Scholten, J. D., Sands, R. H., Ludwig, M. L., and Matthews, R. G. (1996) *Biochemistry* **35,** 2464–2475
38. Shibata, N., Masuda, J., Tobimatsu, T., Toraya, T., Suto, K., Morimoto, Y., and Yasuoka, N. (1999) *Structure Fold Des.* **7,** 997–1008

Importance of the Histidine Ligand to Coenzyme B_{12} in the Reaction Catalyzed by Methylmalonyl-CoA Mutase

Monica Vlasie, Shantanu Chowdhury and Ruma Banerjee

J. Biol. Chem. 2002, 277:18523-18527.
doi: 10.1074/jbc.M111809200 originally published online March 13, 2002
