# Assembly and Protection of the Radical Enzyme, Methylmalonyl-CoA Mutase, by Its Chaperone†

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ABSTRACT: MeaB is a recently described P-loop GTPase that plays an auxiliary role in the reaction catalyzed by the radical $B_{12}$ enzyme, methylmalonyl-CoA mutase. Defects in the human homologue of MeaB result in methylmalonic aciduria, but the role of this protein in coenzyme $B_{12}$ assimilation and/or utilization is not known. Methylmalonyl-CoA mutase catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA that uses reactive radical intermediates that are susceptible to oxidative inactivation. In this study, we have examined the influence of MeaB on the kinetics of the reaction catalyzed by methylmalonyl-CoA mutase and on the thermodynamics of cofactor binding. MeaB alone has a modest effect on the affinity of the mutase for the 5′-deoxyadenosylcobalamin (AdoCbl) cofactor, increasing it 2-fold from 404 ± 71 to 210 ± 22 nM. However, in the presence of GDP, the affinity for the cofactor decreases 5-fold to 1.89 ± 0.33 $\mu$M, while in the presence of guanosine 5′($\beta-\gamma$ imino)triphosphate, a nonhydrolyzable analogue of GTP, the binding of AdoCbl to the mutase is not detected. Protection against oxidative inactivation of the mutase by MeaB is dependent upon the presence of nucleotides with the MeaB/GDP and MeaB/GTP complexes decelerating the rate of formation of oxidized cofactor by 3- and 15-fold, respectively. This study suggests that MeaB functions in the GTP-dependent assembly of holomethylmalonyl-CoA mutase and subsequent protection of radical intermediates during catalysis.

A common step in the catabolism of branched-chain amino acids, odd-chain fatty acids, and cholesterol is the isomerization of methylmalonyl-CoA to succinyl-CoA, catalyzed by 5′-deoxyadenosylcobalamin (AdoCbl[1] or coenzyme $B_{12}$)-dependent methylmalonyl-CoA mutase (*1*, *2*). While mammals lack the ability to synthesize AdoCbl de novo, they retain the capacity to assimilate the cofactor into an active form to support enzymatic functions. The pathway for intracellular trafficking and utilization of $B_{12}$ is surprisingly complex in mammals despite the utilization of this cofactor by only two known enzymes: the cytoplasmic enzyme, methionine synthase, and the mitochondrial enzyme, methylmalonyl-CoA mutase (*3*, *4*). Much of what is known about the components of intracellular $B_{12}$ trafficking derive from studies of patients with inborn errors in cobalamin metabolism who are differentiated into distinct genetic complementation groups: *cbl*A−G and *mut* (*5*, *6*). In recent years, the two genetic loci corresponding to the mitochondrial or mutase-specific branch of the pathway have been identified that support the function of methylmalonyl-CoA mutase (*mut*) (*7*) and encode adenosyltransferase (*cbl*B) (*8*, *9*) and a GTP-binding protein of unknown function (*cbl*A) (*10*). Mutations in the mitochondrial-specific genes involved in $B_{12}$ metabolism lead to methylmalonic aciduria, an inherited defect that can be fatal (*11*).

Orthologues of the *cbl*A gene are widespread in genomes that encode methylmalonyl-CoA mutase, including bacteria, archaea, and eukarya (*12*). In fact, the strong operonic association between methylmalonyl-CoA mutase and the bacterial orthologue *mea*B led to the identification of the human gene and to mutations in this gene in *cbl*A patient cell lines (*10*). The conservation of *mea*B orthologues in organisms that harbor methylmalonyl-CoA mutase suggests a common auxiliary function for this protein in the context of the mutase. Hence, elucidation of the function of the bacterial protein, MeaB, is likely to provide insights into the human homologue and the specific functional deficit in the *cbl*A class of methylmalonic aciduria.

On the basis of the similarity between *cbl*A and the bacterial gene, *arg*K, that encodes a transporter for basic amino acids, arginine, lysine, and ornithine, it was suggested that methylmalonic aciduria linked to the complementation group *cbl*A, denoted MMAA, may be involved in the mitochondrial translocation of $B_{12}$ (*10*). However, a recent study on *Methylobacterium extorquens* AM1 *mea*B, a bacterial orthologue of *cbl*A, revealed that its disruption in the Δ*mea*B::kan strain results in undetectable methylmalonyl-CoA mutase activity even when exogenous AdoCbl is added to the cell extract (*13*). In contrast, the double mutants, Δ*mea*BΔ*mea*D::kan and Δ*mea*BΔ*epm*::kan, lacking an adenosyltransferase (*mea*D) and methylmalonyl-CoA epimerase (*epm*) and consequently deficient in a functional cofactor or substrate respectively, support partial restoration of methylmalonyl-CoA mutase activity in the presence of exogenous AdoCbl. Thus, these double mutants that do not support

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[1] Abbreviations: AdoCbl, 5′-deoxyadenosylcobalamin; $OH_2$Cbl, aquocob(III)alamin; MMAA, methylmalonic aciduria linked to the complementation group *cbl*A; GMPPNP, guanosine 5′($\beta-\gamma$ imino)-triphosphate.

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mutase activity in vivo retain active apomutase albeit at low levels (~17 and 28% relative to wild-type strains lacking *epm* and *mea*D, respectively). This result was interpreted as evidence that *Mea*B protects methylmalonyl-CoA mutase from suicide inactivation (*13*). Furthermore, MeaB forms a stable complex with methylmalonyl-CoA mutase that can be separated on a native gel (*13*, *14*) and activates methylmalonyl-CoA mutase ~3-fold, although the mechanism of this allosteric regulation is unknown (*13*).

MeaB is a member of the G3E subfamily of P-loop GTPases (*15*), which includes other chaperones, viz. HypB (*16*), UreG (*17*, *18*), and CooC (*19*) that are involved in the assembly of target metalloenzymes (nickel hydrogenase, urease, and CO dehydrogenase, respectively) but whose precise functions are not known. Similar to these chaperones, MeaB exhibits low intrinsic GTPase activity, which is enhanced >100-fold in the presence of methylmalonyl-CoA mutase (*14*). MeaB binds guanosine 5′($\beta-\gamma$ imino)triphosphate (GMPPNP), a nonhydrolyzable analogue of GTP, and GDP with similar and relatively low affinity in the micromolar range. The energetics of the interaction between methylmalonyl-CoA mutase and MeaB have been characterized recently by isothermal titration calorimetry (*14*). They have provided insights into how the presence of the $B_{12}$ cofactor in methylmalonyl-CoA mutase and the identity of the nucleotide bound to MeaB modulate the interaction between these proteins. Thus, dependent upon the combination of the nucleotide and mutase form, the dissociation constant for the binding of methylmalonyl-CoA mutase and MeaB ranges from 34 ± 4 to 524 ± 66 nM. While the apomutase does not show a preference for the GDP versus the GMPPNP bound form of MeaB, the holomutase binds MeaB 15-fold more tightly when GMPPNP is bound versus GDP (*14*).

In this study, we have characterized the influence of MeaB on the kinetics of the reaction catalyzed by methylmalonyl-CoA mutase and demonstrated that MeaB modulates AdoCbl binding to apomutase and that cofactor binding is not detected in the presence of a nonhydrolyzable analogue of GTP, GMPPNP. Furthermore, MeaB protects the radical enzyme from oxidative inactivation but only in the presence of nucleotides. These in vitro studies suggest that the chaperone plays a role both in the assembly of the holomutase and in subsequently protecting it against inactivation.

## MATERIALS AND METHODS

*Materials.* AdoCbl, GTP, GMPPNP, GDP, and ATP were purchased from Sigma. Methylmalonic acid was purchased from Fluka and converted to methylmalonyl-CoA using malonyl-CoA synthetase as described previously (*20*). [$^{14}$C]-$CH_3$-malonyl-CoA (56 Ci/mol) was purchased from New England Nuclear. All other chemicals were reagent-grade and were used without further purification.

*Purification of MeaB and Methylmalonyl-CoA Mutase.* Recombinant *M. extorquens* AM1 MeaB and methylmalonyl-CoA mutase were purified from *Escherichia coli* using the expression plasmids developed in the Lidstrom laboratory (*13*) as described previously (*14*).

*Methylmalonyl-CoA Mutase Assays.* The specific activity of methylmalonyl-CoA mutase was determined in the radiolabeled assay at 37 °C as described previously (*21*). A total of 1 unit of activity corresponds to the formation of 1 $\mu$mol of succinyl-CoA min$^{-1}$. The kinetic parameters for the wild-type enzyme with or without 200 nM MeaB and 1 mM Mg-GDP were determined in the presence of varying concentrations of (*R*,*S*)-[$^{14}$C]-methylmalonyl-CoA (90−2700 $\mu$M). The $K_{act}$ for MeaB (±1 mM Mg-GDP) was determined in the presence of 2.5 mM (*R*,*S*)-[$^{14}$C]-methylmalonyl-CoA by varying the concentration of MeaB (2−200 nM). The concentration of AdoCbl (50 $\mu$M) was kept constant in these assays.

*Detection of Cob(II)alamin under Steady-State Conditions in the Presence of MeaB.* The formation of cob(II)alamin under steady-state conditions was monitored by UV−vis spectroscopy (Cary 100 Bio, Varian) under aerobic conditions at 20 °C. Methylmalonyl-CoA was added to a final concentration of 7−10 mM to the holoenzyme (70 $\mu$M) in 50 mM potassium phosphate at pH 7.5 containing 85 $\mu$M MeaB (±1 mM Mg-GDP or Mg-GMPPNP). The amount of cob(II)alamin formed was estimated from the decrease in absorbance at 525 nm [$\Delta\epsilon_{525\ nm} = -4.8$ mM$^{-1}$ cm$^{-1}$ (*22*)].

*Influence of MeaB on the Oxidation of Cob(II)alamin.* The oxidation of cob(II)alamin in the absence or presence of MeaB (complex 1:1 with methylmalonyl-CoA mutase) was monitored by UV−vis spectroscopy by following the conversion of enzyme-bound cob(II)alamin to aquocob(III)-alamin ($OH_2$Cbl). To 20−50 $\mu$M holoenzyme in 50 mM potassium phosphate at pH 7.5, a solution of methylmalonyl-CoA (7−10 mM final concentration) was added, and the oxidation of the intermediate cob(II)alamin was monitored at 20 °C. To determine the influence of MeaB, a 1.5-fold excess of MeaB over methylmalonyl-CoA mutase was employed in the reaction mixture. When used, Mg-GDP, Mg-GTP, or Mg-GMPPNP were added to the reaction mixture at a final concentration of 1 mM. The rate of inactivation was determined by plotting the absorbance at 351 nm versus time. The data were well-fitted to a single-exponential function, $A = A_0 + a(1 - e^{-kt})$, where $A$ is the absorbance at 351 nm, $k$ is the rate constant for inactivation, and $A_0$ is the initial absorbance of the radical at 351 nm.

*Isothermal Titration Calorimetry*. The effect of MeaB upon the binding of AdoCbl to methylmalonyl-CoA mutase was measured as follows. The enzyme (7−20 $\mu$M of methylmalonyl-CoA mutase alone or in a 1:1 complex with MeaB) was titrated with 29 aliquots (10 $\mu$L) of a 90−250 $\mu$M solution of AdoCbl in 50 mM potassium phosphate at pH 7.5 and 300 mM KCl at 20.0 ± 0.1 °C. When used, nucleotides (Mg-GDP or Mg-GMPPNP) were added to a final concentration of 1 mM. It should be noted that titrations performed in phosphate and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffers yielded slightly different binding values for the enthalpy change ($\Delta\Delta H° \sim$ 1 kcal/mol). The calorimetric signals were integrated, and data were analyzed with Microcal ORIGIN software using a single-site-binding model to determine the equilibrium association constant, $K_A$ ($=1/K_d$), and the binding enthalpy, $\Delta H°$. The Gibbs free energy of binding, $\Delta G°$, and the entropic contribution to the binding free energy, $-T\Delta S°$, were calculated using eqs 1 and 2

$$\Delta G° = -RT \ln K_A \quad (1)$$

$$\Delta G° = \Delta H° - T\Delta S° \quad (2)$$

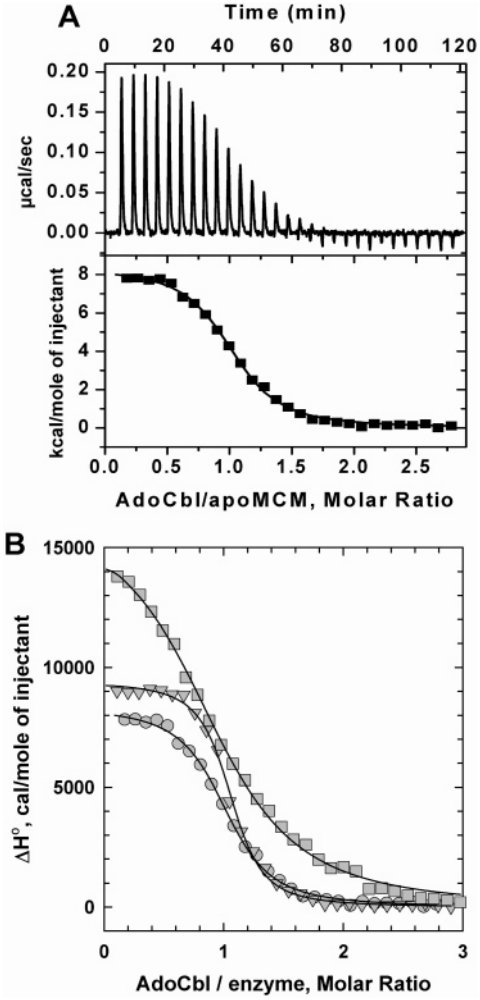


FIGURE 1: Binding isotherms for the binding of AdoCbl to methylmalonyl-CoA mutase versus its complex with MeaB. (A) Representative isothermal titration calorimetry data set for the binding of AdoCbl (108 $\mu$M stock solution) to 8.7 $\mu$M apomethylmalonyl-CoA mutase in 50 mM phosphate buffer at pH 7.5 and 300 mM KCl at 20 °C. (B) Titration curves for the binding of AdoCbl to apomethylmalonyl-CoA mutase alone (●), in the presence of MeaB (▼), and MeaB/GDP (■) in 50 mM phosphate buffer at pH 7.5 and 300 mM KCl at 20 °C. Data were fitted to a single-binding-site model and yielded the parameters given in Table 1.

Table 1: Effect of MeaB on the $K_{d\ AdoCbl}$ for Methylmalonyl-CoA Mutase[a]

| MeaB | nucleotide | $K_{d\ AdoCbl}$ (nM) | $\Delta H°$ (kcal/mol) | $T\Delta S°$ (kcal/mol) | $\Delta G°$ (kcal/mol) |
|---|---|---|---|---|---|
| − | none | 404 ± 71 | 7.8 ± 0.5 | 16.4 ± 0.6 | −8.6 ± 0.1 |
| + | none | 210 ± 22 | 9.1 ± 0.8 | 18.1 ± 0.8 | −8.9 ± 0.2 |
| + | GDP | 1890 ± 330 | 16.0 ± 0.6 | 23.6 ± 0.6 | −7.7 ± 0.1 |

[a] The thermodynamic parameters are an average of at least two independent experiments [±standard deviation (SD)] performed at 20 °C.

## RESULTS

*Effect of MeaB upon the Binding of AdoCbl to Methylmalonyl-CoA Mutase.* The energetics of AdoCbl binding to the *M. extorquens* mutase was monitored by isothermal titration calorimetry (Figure 1 and Table 1). Binding of the cofactor is accompanied by a $\Delta G°$ of −8.6 ± 0.1 kcal/mol and is entropically driven ($T\Delta S° = 16.4 \pm 0.6$ kcal/mol). The $K_d$ for AdoCbl in the presence of MeaB alone (210 ± 22 nM) was ~2-fold lower than the value obtained in its absence (404 ± 71 nM) and resulted from small changes in the contributions of both the entropic and enthalpic terms. However, the presence of MeaB/GDP resulted in a 5-fold higher $K_d$ (1.89 ± 0.33 $\mu$M) that resulted from significant changes in both the enthalpic and entropic terms. Interestingly, in the presence of GMPPNP, a nonhydrolyzable analogue of GTP, no heat release was observed (data not shown), suggesting that GTP hydrolysis may be required for binding of AdoCbl to the methylmalonyl-CoA mutase−MeaB/GTP complex.

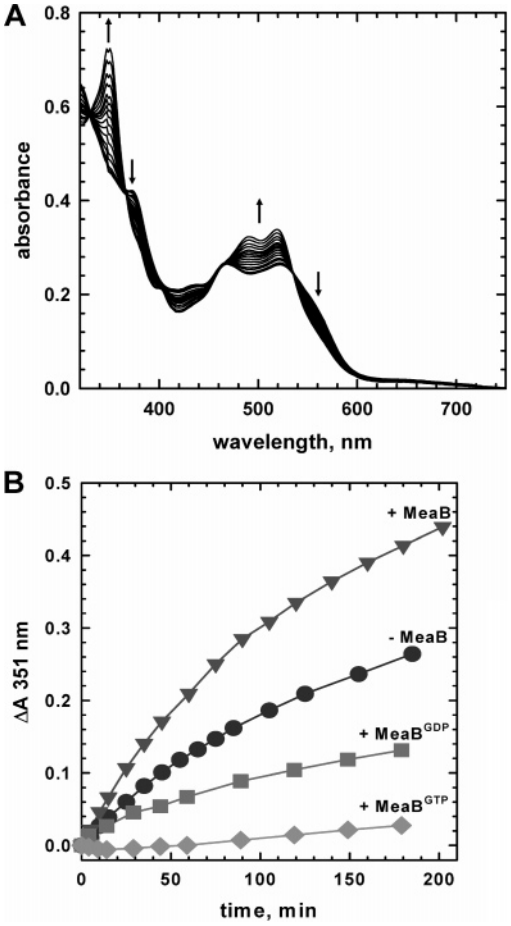


FIGURE 2: Effect of MeaB on the rate of oxidation of mutase-bound cob(II)alamin. (A) Slow oxidation of cob(II)alamin generated by incubating holomethylmalonyl-CoA mutase (44 $\mu$M) with 7 mM methylmalonyl-CoA in 50 mM phosphate buffer at pH 7.5 in the dark at 20 °C. The spectra were recorded between 5 and 190 min. The rate of oxidation as monitored by an increase in absorbance at 351 nm is 0.0072 $min^{-1}$. (B) Presence of nucleotides enables MeaB to protect methylmalonyl-CoA mutase from oxidative inactivation. The rate of $OH_2Cbl$ formation was monitored at 351 nm. The $k_{obsd}$ for the mutase ranged from 0.0093 $min^{-1}$ in the presence of apo-MeaB to 0.0032 $min^{-1}$ in the presence of MeaB/GDP and 0.0006 $min^{-1}$ in the presence of MeaB/GTP (or GMPPNP).

*Effect of MeaB upon the Inactivation of Methylmalonyl-CoA Mutase.* On the basis of genetic studies using deletion mutants of *M. extorquens*, it has been postulated that MeaB could function in protecting the mutase from inactivation (*13*). During steady-state turnover, the cofactor shuttles between the 5′-deoxyadenosylcob(III)alamin and the cob(II)alamin states. The latter contains an unpaired electron and is susceptible to oxidative inactivation (Figure 2A) that leads to the formation of $OH_2Cbl$ at a rate of 0.0072 ± 0.0003 $min^{-1}$ at 20 °C. The addition of stoichiometric

Table 2: Effect of MeaB on the Kinetic Parameters of Methylmalonyl-CoA Mutase[a]

| parameter | MCM | MCM/MeaB | MCM/MeaB/GDP |
|---|---|---|---|
| $k_{cat}$ ($s^{-1}$) | 132 ± 16 | 255 ± 17 | 237 ± 12 |
| $K_{M\ MCoA}$ ($\mu$M) | 86 ± 13 | | 152 ± 13 |
| $k_{cat}/K_M$ ($M^{-1}\ s^{-1}$) | $1.53 \times 10^6$ | | $1.56 \times 10^6$ |
| $K_{d\ AdoCbl}$ (nM) | 404 ± 71 | 210 ± 22 | 1890 ± 330 |
| cob(II)alamin (%)[b] | 48 ± 5 | 80 ± 5 | |
| $K_{act(MeaB)}$ (nM) | | 4.4 ± 1.2 | 14.2 ± 3.8 |

[a] The kinetic parameters were determined at 37 °C and represent the average of two independent experiments (±SD). $K_d$ values were determined by isothermal titration calorimetry at 20 °C and are the average of at least two independent experiments (±SD). [b] The data represent the average of three independent experiments in phosphate buffer as described under Materials and Methods. In HEPES buffer, cob(II)alamin represented 22.5 ± 3 and 44 ± 5% of the total bound cofactor in the absence and presence of MeaB, respectively.

amounts of MeaB had a small effect on accelerating $k_{obsd}$ to 0.0093 ± 0.0003 $min^{-1}$ (Figure 2B). Interestingly, a protective effect was seen when a MeaB/nucleotide complex was added to the mutase versus MeaB alone. The presence of GTP had a significant effect on the inactivation rate for the mutase, decreasing it ~15-fold compared to MeaB alone; GDP had a smaller effect (~3-fold). When GMPPNP was used, the same protective effect was observed as with GTP.

*Effect of MeaB (±Nucleotides) on the Steady-State Kinetic Parameters of Methylmalonyl-CoA Mutase.* Because the GTPase activity of MeaB is enhanced >100-fold in the presence of the mutase (*14*), we investigated the effect of the MeaB/GDP complex on the kinetic parameters for the mutase under steady-state conditions using the radiolabeled assay as described under Materials and Methods (Table 2). MeaB/GDP increased the $k_{cat}$ for the mutase 1.8-fold and increased $K_M$ for the substrate by exactly the same magnitude (Figure 3A). Consequently, MeaB/GDP had no effect on the catalytic efficiency, $k_{cat}/K_M$, of the mutase. The dependence of the specific activity on the concentration of MeaB alone yielded an estimate for $K_{act[MeaB]}$ of 4.4 ± 1.2 nM, which increased to 14.2 ± 3.8 nM in the presence of MeaB/GDP (Figure 3B).

*Effect of MeaB on the Steady-State Distribution of Cofactor Forms Bound to Methylmalonyl-CoA Mutase.* Kinetic analysis of *Propionibacterium shermanii* methylmalonyl-CoA mutase indicates that the reaction is limited by the product release step (*23*, *24*). This is consistent with the predominance of AdoCbl (80%) under steady-state turnover conditions over cob(II)alamin (20%), because the ternary enzyme/AdoCbl/product complex accumulates prior to the rate-determining product-release step (*23*). Assuming that the kinetics of methylmalonyl-CoA mutase from *M. extorquens* AM1 are similar, the change in $k_{cat}$ effected by MeaB is expected to decrease the barrier for the product-release step, which in turn would lead to a redistribution of the AdoCbl and cob(II)alamin species observed during enzyme-monitored turnover. To test this prediction, the spectrum of the *M. extorquens* AM1 methylmalonyl-CoA mutase was monitored under steady-state turnover conditions (Figure 4). The ratio of cob(II)alamin/AdoCbl was found to be buffer-dependent and was 0.23:0.77 in HEPES buffer and 0.48:0.52 in phosphate buffer at the same pH. The presence of MeaB alone increased the proportion of cob(II)alamin ~2-fold to 44 and 80% in HEPES and phosphate buffer, respectively.

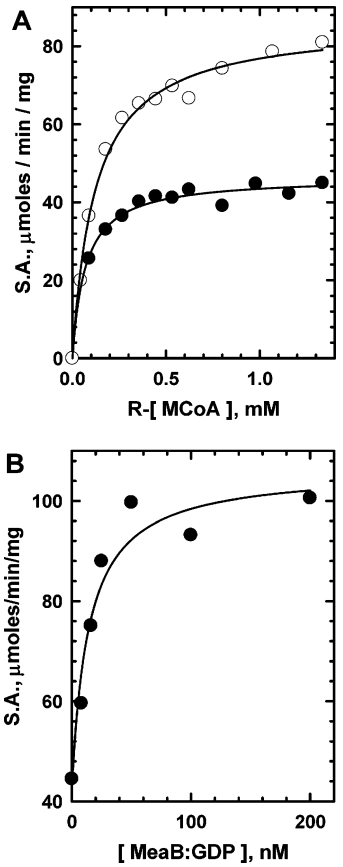


FIGURE 3: MeaB increases methylmalonyl-CoA mutase activity. (A) Michaelis−Menten analysis of the dependence of the specific activity of methylmalonyl-CoA mutase in the absence (●) or presence (○) of 200 nM MeaB/GDP on the concentration of (*R*)-methylmalonyl-CoA (45−1350 $\mu$M) in 50 mM phosphate buffer at pH 7.5 and 37 °C. The kinetic parameters derived from this analysis are reported in Table 2. (B) Dependence of the specific activity of methylmalonyl-CoA mutase on the concentration of MeaB/GDP in the presence of 1.3 mM (*R*)-methylmalonyl-CoA in 50 mM phosphate buffer at pH 7.5 and 37 °C. The $K_{act}$ derived from this analysis is reported in Table 2.

In contrast to MeaB alone, the presence of the MeaB/GDP or MeaB/GMPPNP complex decreased the relative concentration of cob(II)alamin and elicited spectral perturbations. A red shift was observed in the cob(II)alamin spectrum from 465 to 470 nm (MeaB/GDP) and 483 nm (MeaB/GMPPNP), respectively. In addition, a decrease in absorption at 525 nm was seen in the mutase−MeaB/GMPPNP complex in the absence of the substrate (not shown). These spectral changes make it difficult to precisely quantify the relative concentrations of cob(II)alamin and AdoCbl in the presence of MeaB/nucleotides, but it appears to be intermediate between the mutase and mutase/MeaB reaction mixtures.

## DISCUSSION

The discovery that MMAA represents the *cbl*A locus of inborn errors of cobalamin metabolism and leads to a functional deficiency of methylmalonyl-CoA mutase with consequent organic aciduria (*10*, *25*) raises questions about the role of this auxiliary protein in the function of the mitochondrial radical enzyme. Furthermore, the presence of this gene in bacterial operons that encode methylmalonyl-CoA mutase (*12*, *13*) suggests a conservation of function

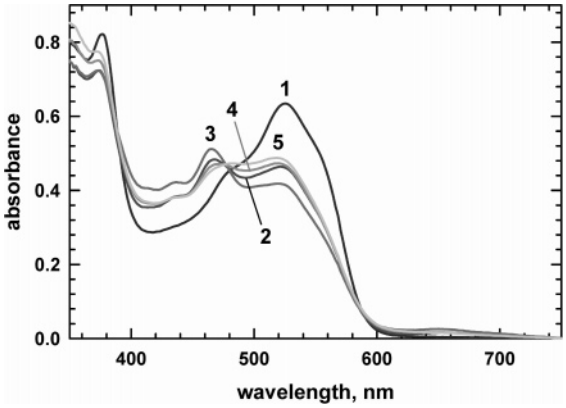


FIGURE 4: Formation of cob(II)alamin under steady-state conditions with or without MeaB. UV−vis absorption spectra of holomethylmalonyl-CoA mutase (70 $\mu$M, spectrum 1) in 50 mM phosphate buffer at pH 7.5 and 20 °C and following the addition of 8 mM methylmalonyl-CoA (spectrum 2). The remaining traces were obtained by the addition of 85 $\mu$M MeaB alone (spectrum 3), MeaB/GDP (spectrum 4), or MeaB/GMPPNP (spectrum 5) to the mutase, and the reaction was initiated by the addition of the substrate under conditions described for spectrum 1. Nucleotides, when employed, were present at a concentration of 1 mM.

between organisms. MeaB, a bacterial orthologue of MMAA, exists as a dimer that forms a stable complex with methylmalonyl-CoA mutase (*13*, *14*). The energetics of the association of nucleotides to MeaB and of the modulation of the interaction between MeaB and the mutase by both nucleotides and $B_{12}$ have been described recently (*14*). The formation of the MeaB−methylmalonyl-CoA mutase complex influences the GTPase activity of MeaB, which is stimulated >100-fold. In this study, we have examined the effect of MeaB ± nucleotides on binding of AdoCbl on the catalytic properties of the mutase and on the kinetics of inactivation under steady-state turnover conditions.

Binding of AdoCbl to methylmalonyl-CoA mutase is entropically driven, which is consistent with the structural reorganization that is predicted to accompany cofactor binding with a concomitant release of ordered water molecules from the protein. The presence of MeaB alone has a modest effect on the energetics of AdoCbl binding and enhances the affinity for the cofactor by 2-fold, whereas the MeaB/GDP complex diminishes the affinity for the cofactor 5-fold (Table 1). A substantial conformational change is predicted to accompany complex formation between MeaB/GDP and apomethylmalonyl-CoA mutase with an estimated 5300 Å$^2$ of surface area becoming buried upon complex formation involving the structural reorganization of ~49 residues (*14*). In addition, dynamic light scattering studies indicate that the Stokes radius for MeaB decreases from 4.4 nm in the absence of nucleotides to 3.3 nm in their presence, consistent with a conformational change that leads to a more compact structure (*14*). This reorganization may affect the interaction between MeaB and the mutase and, in turn, the cofactor-binding site, thereby modulating the affinity of AdoCbl. Interestingly, in the presence of GMPPNP, no heat release was observed by isothermal titration calorimetry, suggesting that GTP hydrolysis is required for cofactor binding to the mutase−MeaB/GTP complex. This is consistent with our hypothesis that MeaB functions to gate cofactor binding to the mutase in a GTP-dependent fashion (*6*).

Methylmalonyl-CoA mutase uses AdoCbl to catalyze a chemically challenging carbon skeleton rearrangement reaction that proceeds via radical intermediates. The latter are highly reactive and must be protected from adventitious interception. While the eliminase subclass of isomerases have chaperones that catalyze an exchange of the inactive $OH_2$-Cbl cofactor for the active AdoCbl form, in an ATP-dependent reaction (*26*), such chaperones do not appear to exist in operons encoding the structural gene for methylmalonyl-CoA mutase. Because the formation of $OH_2$Cbl leads to irreversible accumulation of inactive methylmalonyl-CoA mutase, a strategy for averting or minimizing this side reaction must have evolved. In this context, the strong operonic association between methylmalonyl-CoA mutase and MeaB and its orthologues in bacterial genomes (*10*, *12*), and the genetic evidence that suggests protection of the mutase by MeaB (*13*), suggest that this P-loop GTPase is a mutase chaperone.

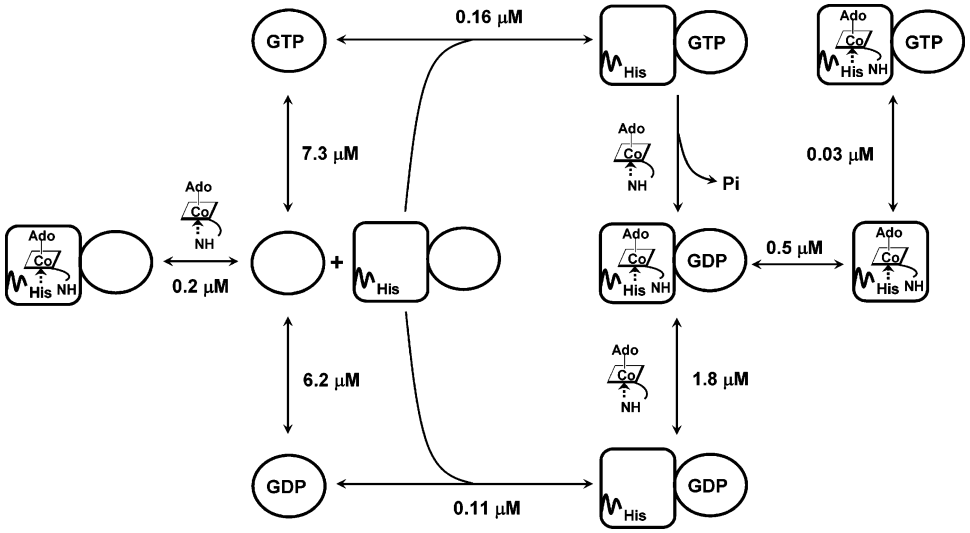


FIGURE 5: Model summarizing the interactions between MeaB and mutase in the presence of different combinations of nucleotides and cofactor. The square represents methylmalonyl-CoA mutase, and the circle represents MeaB. The dissociation constants included in this scheme that are not from this study have been reported previously (*14*). It is assumed that GMPNP is a good mimic for GTP for the binding constants reported in this model.

Oxidation of cob(II)alamin to $OH_2$Cbl can be readily monitored by UV−vis absorption spectroscopy (Figure 2A), and a slow loss of active cofactor at a rate of 0.0072 $min^{-1}$ is observed. Although apo-MeaB did not have a protective effect and in fact was found to enhance the rate of cofactor oxidation slightly, the addition of either GDP or GTP afforded significant protection (3- and 15-fold, respectively) to the mutase from inactivation (Figure 2B). Intriguingly, protection in the presence of nucleotides was apparently not correlated with the steady-state concentration of cob(II)alamin, the intermediate that is oxidized to $OH_2$Cbl. Thus, while the concentration of cob(II)alamin was ~2-fold higher in the presence of MeaB, it was essentially unchanged in the presence of MeaB/nucleotides. However, both nucleotides elicited a discernible perturbation in the spectrum of cob(II)alamin, corresponding to the $\pi-\pi^*$ transitions in the corrin ring (*27*), indicating a change in the environment of the cofactor in the presence of MeaB/nucleotide complexes (Figure 4) that is associated with the shielding of the cofactor from oxidative interception. The observation that GMPPNP confers the same protective effect as GTP suggests that binding rather than hydrolysis of the nucleotide is important. Thus, in contrast to the chaperones for $B_{12}$-dependent eliminases (*26*), the protective effect afforded by MeaB/nucleotide on methylmalonyl-CoA mutase does not appear to be driven by the energy of nucleotide hydrolysis but rather by conformational effects driven by binding energy.

The effect of MeaB (±GDP) on the kinetic parameters of the mutase-catalyzed reaction reported earlier (*13*) and further characterized in this study is unlikely to be physiologically significant. Thus, MeaB/GDP enhances the turnover number of the mutase 1.8-fold, while also increasing the $K_M$ for its substrate. Hence, there is no net effect on $k_{cat}/K_M$, conditions under which the mutase is expected to operate in the cell.

On the basis of the thermodynamic parameters that are presently available for the interaction of MeaB and methylmalonyl-CoA mutase, we propose the model shown in Figure 5. MeaB exhibits a similar affinity for GDP and GTP with $K_d$ values of 6.2 ± 0.7 and 7.3 ± 0.6 $\mu$M, respectively (*14*). Although the affinity of apomethylmalonyl-CoA mutase for MeaB/nucleotide is apparently insensitive to the presence of GDP ($K_d$ = 0.11 ± 0.02 $\mu$M) versus GMPPNP ($K_d$ = 0.16 ± 0.02 $\mu$M) (*14*), MeaB is expected to exist predominantly in the GTP-bound state under physiological conditions, where the concentration of this nucleotide is relatively high [~0.9 mM (*28*)]. Binding of AdoCbl to the apomutase−MeaB/GTP complex appears to require hydrolysis of GTP to give the presumed product, holomutase−MeaB/GDP, which in turn, confers a protective advantage to the mutase under catalytic turnover conditions (Figure 2). Alternatively, dissociation of the holomutase−MeaB/GDP complex ($K_d$ = 0.52 ± 0.06 $\mu$M) and reassociation of the holomutase with MeaB/GMPPNP ($K_d$ = 0.034 ± 0.004 $\mu$M) would yield a high-affinity complex that is even more effective in protecting against oxidative inactivation of radical intermediates.

In summary, the MeaB−methylmalonyl-CoA mutase complex elicits bidirectional responses with each protein, influencing the properties of the other. We have previously characterized the effect of the mutase on nucleotide binding and the kinetics of hydrolysis by MeaB (*14*). On the basis of this study, we propose that MeaB has dual functions. First, it is involved in the binding of AdoCbl to apomethylmalonyl-CoA mutase in a GTPase-dependent step, and second, it protects the radical intermediates formed during the reaction catalyzed by the mutase in a GTP-independent manner.

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