Energetics of Interaction between the G-protein Chaperone, MeaB, and B$_{12}$-dependent Methylmalonyl-CoA Mutase*

Dominique Padovani, Tetyana Labunska, and Ruma Banerjee

From the Redox Biology Center and the Biochemistry Department, University of Nebraska, Lincoln, Nebraska 68588-0664

MeaB is an auxiliary protein that supports the function of the radical B$_{12}$-dependent enzyme, methylmalonyl-CoA mutase, although its precise role is not understood. Mutations in the human homolog of MeaB, MMAA, lead to methylmalonic aciduria, an inborn error of metabolism that can be fatal. To obtain insights into the function of this recently discovered protein, we have characterized the entropic and enthalpic contributions to $\Delta G_{assoc}$ for complexation of MeaB (in the presence and absence of nucleotides) with methylmalonyl-CoA mutase (in the presence and absence of cofactor). The dissociation constant for binding of methylmalonyl-CoA mutase and MeaB ranges from $34 \pm 4$ to $524 \pm 66$ nM, depending on the combination of nucleotide and mutase form. Holomutase binds MeaB 15-fold more tightly when the nonhydrolyzable GTP analog, GMPPNP, is bound versus GDP. In contrast, the apomutase binds MeaB with similar affinity in the presence of either nucleotide. Our studies reveal that a large structural rearrangement accompanies interaction between these proteins and buries between $-4000$ and $8600$ Å$^2$ of surface area, depending on the combination of ligands in the active sites of the two proteins. Furthermore, we demonstrate that MeaB binds GTP and GDP with similar affinity ($K_d$ of $7.3 \pm 1.9$ and $6.2 \pm 0.7$ μM, respectively at 20 °C) and has low intrinsic GTPase activity ($-0.04$ min$^{-1}$ at 37 °C), which is stimulated $\sim 100$-fold by methylmalonyl-CoA mutase. These studies provide insights into the energetics of interaction between the radical enzyme methylmalonyl-CoA mutase and MeaB, which are discussed.

In recent years a number of auxiliary P-loop GTPases that function as chaperones in the assembly of metal cofactors in target enzymes have been described (1). Their GTPase activity was initially inferred from sequence analysis that revealed the presence of signature motifs associated with this superfamily (2) and include a Walker A or P-loop and Walker B motifs, an aspartate residue involved in Mg$^{2+}$ binding, and a GTP-binding (N/T)KXD sequence. Members of this family include UreG, which is involved in the assembly of the nickel-based metalocenter in urease (3, 4); HypB, which is required for nickel hydrogenases (5); CooC for CO dehydrogenase (6); and MeaB for B$_{12}$-dependent methylmalonyl-CoA mutase (7). Although many of these proteins have since been demonstrated to possess low GTPase activity, their precise function in the assembly of their respective metalloprotein targets remains to be resolved.

The most recently described member of this subfamily of GTPases is MeaB, which is strongly associated in bacterial operons with methylmalonyl-CoA mutase (8), a B$_{12}$-dependent isomerase that catalyzes the rearrangement of methylmalonyl-CoA to succinyl-CoA (9, 10). An ortholog of MeaB, MMAA, is found in humans and is the locus of mutations associated with the cblA class of inborn errors of cobalamin disorders that lead to methylmalonic aciduria (11, 12). In humans, the cblA gene product and methylmalonyl-CoA mutase are localized in the mitochondrion. Based on the similarity between cblA and the *Escherichia coli* argK gene that encodes a lysine/ornithine/arginine transporter, it was proposed that cblA could be involved in the mitochondrial translocation of B$_{12}$ (11). However, a recent study on MeaB from *Methylobacterium extorquens* AM1 suggests that it may be involved in protecting methylmalonyl-CoA mutase, a radical enzyme, from inactivation (7).

A second mitochondrial protein that plays an auxiliary role for the methylmalonyl-CoA mutase-catalyzed reaction is adenosyltransferase, the locus of the *cblB* class of genetic defects (13, 14). Adenosyltransferase catalyzes the synthesis of adenosylcobalamin (AdoCbl), the active cofactor form utilized by methylmalonyl-CoA mutase. It has been postulated that adenosyltransferase is a dual function protein that serves as an enzyme to synthesize AdoCbl and an escort, which delivers it to the mutase (15). It is speculated that MMAA (or MeaB) may gate the transfer of AdoCbl from adenosyltransferase to the mutase in a GTP-dependent manner (Fig. 1). The fusion of MeaB and mutase in a single polypeptide in some organisms (7) suggests that these two proteins influence each other’s catalytic activities. However, the energetics of the interaction between these proteins and the modulation of kinetics in the complex are unknown but are needed to test and refine the current working model.

Clearly the functional elucidation of a highly conserved auxiliary protein for methylmalonyl-CoA mutase that is the locus of disease-causing mutations in humans is an important problem that merits attention. In addition, it is likely that insights into the function of any one of the members of the subfamily of P-loop GTPases that plays an auxiliary role in the function of a target metalloenzyme are likely to shed light on the mechanism of action of the others. In this study, we have characterized the thermodynamic parameters associated with the interaction of MeaB and methylmalonyl-CoA mutase from *M. extorquens* AM1 and demonstrate that the interaction of the two proteins leads to a substantial structural reorganization. Furthermore, we demonstrate that the low intrinsic GTPase activity of MeaB is stimulated $\sim 100$-fold by methylmalonyl-CoA mutase, and the identity of the nucleotide as well as the presence or absence of the B$_{12}$ cofactor modulate the affinity between the two proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**

AdoCbl, GTP, GMPPNP, GDP, cGMP, GMP, AMPPNP, and ATP were purchased from Sigma. Methylmalonic acid was purchased from...
Fluka. [14C]CH₃-malonyl-CoA (56 Ci/mol) was purchased from New England Nuclear. All other chemicals were reagent grade and were used without further purification.

Plasmids

The plasmid vectors pKN13 and pET21d:meaB, containing respectively the M. extorquens AM1 methylmalonyl-CoA mutase and meaB genes, were generous gifts from Mary E. Lidstrom (University of Washington, Seattle, WA).

Enzyme Expression and Purification

The E. coli strain BL21(DE3) was freshly transformed with pKN13 or pET21d:meaB plasmids, and this mixture was used directly to inoculate Luria Bertani medium (100 μl of transformed cells/liter of culture) supplemented with 100 μg/ml ampicillin. The cultures were grown overnight (~12 h) at 37 °C to an A₆₀０ of 0.5 and induced with 0.5 mM isopropyl β-D-thiogalactopyranoside at 30 °C for MeaB or 23 °C for methylmalonyl-CoA mutase and grown for an additional 6~8 h. The proteins were purified as described (7) with the following minor modifications. Purified MeaB was concentrated, exchanged into 50 mM HEPES, pH 8.0, containing 300 mM KCl, 2.5 mM MgCl₂, and 5% glycerol (buffer B), and stored at ~80 °C. For methylmalonyl-CoA mutase, after the nickel-nitrilotriacetic acid column, fractions of interest were pooled, then washed at the same flow rate with 500 ml of buffer A and eluted with a 500-ml gradient from 50 to 250 mM KCl in 50 mM potassium phosphate, pH 7.5. The protein solution was then applied to a 5 × 7.5 cm monoQ column (131 POROS HQ/H) equilibrated at a flow rate of 10 ml/min with buffer A (50 mM potassium phosphate, pH 7.5, 50 mM KCl). The column was then washed at the same flow rate with 100 ml of buffer A and eluted with a 500-ml gradient from 50 to 300 mM potassium phosphate, pH 7.5 over 50 min at the same flow rate. The purified MCM was concentrated, exchanged into 50 mM potassium phosphate, pH 7.5, and stored at ~80 °C. Protein concentration was determined using the Bradford reagent (Bio-Rad) with bovine serum albumin as a standard.

Size Exclusion Chromatography

To determine the oligomeric state of MeaB, ~0.1~0.2 mg of the enzyme was loaded on a 2 × 70-cm Sephacryl 200 column in buffer B at a flow rate of 2 ml/min⁻¹. The column was calibrated using gel filtration standards from Bio-Rad.

Dynamic Light Scattering

These experiments were performed on a DynaPro apparatus (Protein Solutions) equipped with a temperature-controlled microsampler and the Dynamics V6 software. A solution of MeaB (1 mg/ml) in buffer B to which 1 mM GTP or GDP was added as desired, was centrifuged at 4 °C at 15,000 rpm for 30 min and then filtered through a 0.02-μm Anotop 10 filter (Whatman). The solution was then loaded into a quartz cell, and the measurements were performed at 15 °C using 10 sample acquisitions/experiment.

PAGE Analysis of Complex Formation

To analyze complex formation between MeaB and methylmalonyl-CoA mutase, 16 μg of apoenzyme was mixed with 10 μg of MeaB either alone or in the presence of (i) 50 μM AdoCbl or (ii) 50 μM AdoCbl and 1 mM methylmalonyl-CoA at 20 °C in 50 mM HEPES, pH 8.0, 300 mM KCl, 10 mM MgCl₂, 5% glycerol. After a 10-min incubation, the mixtures were analyzed by PAGE under nondenaturing condition in a 4~15% gradient gel (Bio-Rad) over ~10 h at 4 °C. The same experiment was repeated in the presence of 1 mM GDP, GTP or GMPPNP, and no difference was observed in the stoichiometry of complex formation.

Kinetics of MeaB-catalyzed GTP Hydrolysis

The kinetic parameters for the GTPase activity of MeaB (10 μM) were determined in the presence of various concentrations of GTP (50~5000 μM) at 37 °C in 50 mM HEPES, pH 8.0, 300 mM KCl, 10 mM MgCl₂, 5% glycerol. For each GTP concentration, aliquots were taken at intervals (5~120 min), treated with 1 nM trichloroacetic acid (10% v/v), centrifuged, and filtered through a 0.02-μm Anotop 10 filter (Whatman) to remove the precipitated protein. The nucleotides were analyzed by reversed phase chromatography (250 × 4.6 mm Luna C18, 5μ; Phenomenex) eluted isocratically with 4.5% acetonitrile in 10 mM tetrabutylammonium bromide, 75 mM potassium phosphate, pH 6.5, over 30 min at a flow rate of 1 ml/min⁻¹. The eluant was monitored at 254 nm. Under these conditions, GDP and GTP eluted at ~8.7 and 15.0 min, respectively. Control experiments were performed where MeaB was omitted from the reaction mixture. The velocity of GTP hydrolysis was estimated for each concentration of GTP by fitting the data to a single exponential function. The kcat values obtained from this analysis were then plotted against the concentration of GTP, and the kinetic parameters, Km and kcat (under Vmax conditions) were obtained by a Michaelis-Menten analysis of the data set. To determine whether MeaB had ATPase activity, a parallel experiment was performed in which GTP was replaced by 5 mM ATP and analyzed as described above. The effect of methylmalonyl-CoA mutase (apo- or holo-form) on the GTPase activity of MeaB was analyzed by incubating the complex (containing 10 μM of each protein) in 50 mM HEPES, pH 8.0, 300 mM KCl, 10 mM MgCl₂, 5% glycerol with 5 mM GTP at 37 °C. At intervals of 2~30 min, aliquots were removed and analyzed as previously described.

Isothermal Titration Calorimetry

All of the calorimetric binding experiments were performed using a VP-ITC microcalorimeter (1.44-ml cell volume) (Microcal, Inc., Northampton, MA), equipped with a ThermoVac sample degasser and a 300-μl syringe. Prior to performing the titrations, stock solutions of proteins and ligand were filtered through 0.22-μm polyvinylidene difluoride syringe filters (Millipore) and degassed for 10 min. In all experiments, the ligand was added to the protein in the sample cell. The heats of binding were corrected for the heat of ligand dilution by subtraction of the average heat associated with multiple injections of ligand following saturation of the binding sites. Each experiment was performed in duplicate, and the data were analyzed using Microcal ORIGIN software.
Energetics of MeaB-MCM Interaction

Binding of Ligands to MeaB—MeaB (10–100 µM) was titrated with twenty-nine 10-µl aliquots of a 125–1250 µM solution of GDP or GMPPNP in buffer B at temperatures ranging from 5 to 25.0 ± 0.1 °C. To circumvent problems with the GTPase activity of MeaB, the nonhydrolizable analog GMPPNP was employed, and we made the assumption that it bound with affinity similar to that of GTP. The same experiments were repeated with buffer B lacking KCl to assess the importance of electrostatic interactions for effector binding. The calorimetric signals were integrated, and the data were analyzed with Microcal ORIGIN software using a single-site binding model to determine the equilibrium association constant, $K_a$, and the binding enthalpy, $ΔH^o$. The Gibbs free energy of binding, $ΔG^o$, and the entropic contribution to the binding free energy, $−TΔS^o$, were calculated using Equations 1 and 2.

$$ΔG^o = −RT \ln K_a \quad (\text{Eq. 1})$$

$$ΔG^o = ΔH^o − TΔS^o \quad (\text{Eq. 2})$$

No heat release was observed when GMP, cGMP, or ATP was added to MeaB.

Effect of Methylmalonyl-CoA Mutase on Binding of Effectors by MeaB—The binding of nucleotides to the complex of apo- or holo-mutase with MeaB (1:1) containing 8–14 µM of each protein in buffer B was titrated as described above.

Thermodynamics of Methylmalonyl-CoA Mutase-MeaB Complex Formation—In all experiments, 45–180 µM methylmalonyl-CoA mutase (apoenzyme or holoenzyme) was added to 4–15 µM MeaB in the sample cell. GDP or GMPPNP (500 µM) was added both in the syringe and in the cell. Complex formation was titrated in buffer B as described above. The extent of complex rearrangement that occurs during complex formation was estimated as described previously (17). The binding heat capacity change, $ΔC_{H_2O}^{o\text{assoc}}$, a measure of the hydrophobic surface buried during complex formation, was determined by performing the binding experiments at temperatures ranging from 5 to 20 °C ± 0.1 °C. The binding enthalpy change observed at each temperature was then plotted versus temperature, and the data were fitted to a linear function; the slope yielded the value of $ΔC_{H_2O}^{o\text{assoc}}$. Nonpolar ($ΔA_p$) and polar ($ΔA_p$) surface areas (Å²) buried upon complex formation were calculated from the empirical relationships described in Equations 3 and 4.

$$ΔC_{H_2O}^{o\text{assoc}} = 0.45 ΔA_p − 0.26 ΔA_o \quad (\text{Eq. 3})$$

$$ΔH_{H_2O}^{o\text{assoc}} = −8.44 ΔA_p + 31.4 ΔA_o \quad (\text{Eq. 4})$$

where $ΔH_{H_2O}^{o\text{assoc}}$ is the binding enthalpy change at 60 °C. The number of residues that become ordered during binding, $R^o$, was calculated using Equation 5, which describes the net entropy change $ΔS_{H_2O}^{o\text{assoc}}$ which is equal to zero at the characteristic temperature $T_S$.

$$ΔS_{H_2O}^{o\text{assoc}} = 0 = ΔS_{H_2O}^{o\text{sol}}(T_S) + ΔS_{H_2O}^{o\text{fr}} + ΔS_{H_2O}^{o\text{other}} \quad (\text{Eq. 5})$$

The terms contributing to $ΔS_{H_2O}^{o\text{assoc}}$ are the solvation entropy or hydrophobic effect ($ΔS_{H_2O}^{o\text{sol}}(T_S) = 1.35 ΔC_{H_2O}^{o\text{assoc}} \ln (T_S/386)$), an unfavorable entropic term resulting from loss of rotational and translational degrees of freedom for proteins upon binding ($ΔS_{H_2O}^{o\text{fr}} = −50$ entropy units (cal/ (mol⋅K)) for binary protein-protein interactions) and $ΔS_{H_2O}^{o\text{other}}$, which is related to $R^o$ because $R^o = ΔS_{H_2O}^{o\text{other}}/−5.6$ e.u. (17).

RESULTS

Purification and Properties of MeaB and Methylmalonyl-CoA Mutase—Recombinant MeaB containing a C-terminal His tag was obtained using an expression system developed in the Lidstrom laboratory (7) that yielded ~15 mg of highly pure protein/liter of culture. A single band with a molecular mass of ~37 kDa was observed by SDS-PAGE (Fig. 2A), which corresponds to the predicted mass of the polypeptide of 35 kDa. Size exclusion chromatography on a calibrated Sephacryl 200 column yielded an estimated molecular mass of ~76 ± 6 kDa, consistent with it being a homodimer.

Because MeaB has been previously reported to be a monomer (7), dynamic light scattering was used as an additional probe of the oligomerization state of the protein. In the presence of GTP or GDP, >97% of the samples displays a radius of 3.6 nm with an estimated molecular mass of ~67 kDa ($n = 13$ for each experiment; data not shown). Interestingly, in the absence of nucleotides, >99% of the protein displays a radius of 4.4 nm with an estimated molecular mass of 110 kDa ($n = 19$). Thus, nucleotide binding elicits a conformational change that decreases the radius of MeaB, suggesting a more compact structure for the binary complex. The dynamic light scattering data are consistent with MeaB existing as a dimer in solution.

Prior studies from this laboratory have focused on the recombinant Propionibacterium shermanii methylmalonyl-CoA mutase. To avoid the use of interacting proteins from different sources, recombinant M. extorquens AM1 methylmalonyl-CoA mutase was purified using an expression system developed in the Lidstrom laboratory (7). The M. extorquens methylmalonyl-CoA mutase is a heterodimer with subunit molecular masses of 78 and 64 kDa, respectively. Highly pure mutase (136 mg) was isolated from a 6-liter culture (Fig. 2A) and had a specific activity of 54 µmol succinyl-CoA formed/min/mg of protein at 37 °C. This is ~40-fold higher than previously reported (7) and similar to the activity measured in our laboratory for the P. shermanii mutase (18, 19).

Complex Formation Between MeaB and Methylmalonyl-CoA Mutase—MeaB forms a stable complex with methylmalonyl-CoA mutase that
can be resolved on a native polyacrylamide gel. As reported previously (7), two bands corresponding to methylmalonyl-CoA mutase were observed on a native gel (Fig. 2B). Complex formation was monitored in the presence of GMPPNP at an increasing molar ratio of MeaB:methylmalonyl-CoA mutase and was found to saturate at a ratio of ~1:1. This is consistent with the association of one dimer (or two monomers) of MeaB per heterodimer of methylmalonyl-CoA mutase. The addition of 5 mM GTP had no effect on the binding stoichiometry (not shown).

Characterization of Nucleotide Binding to MeaB—The thermodynamic parameters associated with binding of nucleotides to MeaB were assessed by ITC at two ionic strengths (Table 1). The binding of GMPPNP to MeaB is apparently independent of the presence or absence of KCl. Binding of GDP to MeaB is enthalpically driven, whereas both entropic and enthalpic changes contribute favorably to binding of the GTP analog, with entropic factors dominating in the presence of salt. The data were fitted with a binding stoichiometry of one GMPPNP (or GDP) per dimer of MeaB, suggesting half of sites activity. The addition of either GMP, cGMP, or AMPPPNP did not result in measurable heat release.

**TABLE 1**
Thermodynamic parameters for binding of nucleotides to MeaB

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>KCl (mM)</th>
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<tr>
<td></td>
<td>0</td>
<td>5.6 ± 0.4</td>
<td>-5.1 ± 0.7</td>
<td>1.9 ± 0.3</td>
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<tr>
<td>GMPPNP</td>
<td>300</td>
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<td>6.7 ± 0.3</td>
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<tr>
<td>GDP</td>
<td>0</td>
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<td>-13.6 ± 0.5</td>
<td>-5.7 ± 0.4</td>
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<tr>
<td></td>
<td>300</td>
<td>6.2 ± 0.7</td>
<td>-9.2 ± 0.4</td>
<td>-2.2 ± 0.2</td>
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**TABLE 2**
Thermodynamic parameters for binding of nucleotides to MeaB-MCM complexes

<table>
<thead>
<tr>
<th>MCM</th>
<th>MeaB</th>
<th>ΔΗ°</th>
<th>TΔS°</th>
<th>ΔG°</th>
<th>Kd (μM)</th>
<th>ΔC°</th>
<th>ΔH°</th>
<th>ΔS°</th>
<th>ΔG°</th>
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<tr>
<td>Apo-form</td>
<td>GMPPNP</td>
<td>-24.1 ± 0.5</td>
<td>-15.0 ± 0.8</td>
<td>-9.2 ± 0.3</td>
<td>155 ± 24</td>
<td>-1255</td>
<td>3684</td>
<td>4917</td>
<td>80</td>
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<td></td>
<td>GDP</td>
<td>-22.4 ± 0.6</td>
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<td>-9.4 ± 0.2</td>
<td>110 ± 21</td>
<td>-704</td>
<td>2403</td>
<td>2955</td>
<td>49</td>
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<tr>
<td>Holo-form</td>
<td>GMPPNP</td>
<td>-33.0 ± 1.1</td>
<td>-23.0 ± 1.0</td>
<td>-10.0 ± 0.1</td>
<td>34 ± 4</td>
<td>-865</td>
<td>3186</td>
<td>3763</td>
<td>71</td>
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<td></td>
<td>GDP</td>
<td>-27.2 ± 1.2</td>
<td>-18.8 ± 1.2</td>
<td>-8.5 ± 0.2</td>
<td>524 ± 66</td>
<td>-416</td>
<td>1940</td>
<td>2046</td>
<td>35</td>
</tr>
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</table>

**FIGURE 3.** Binding isotherm for complex formation between methylmalonyl-CoA mutase and MeaB. A, calorimetric data for a representative titration of MeaB (4.5 μM) in the presence of 500 μM GMPPNP) and apomethylmalonyl-CoA mutase (52 μM) in buffer B. The top panel shows the raw data in power versus time. The area under each spike is proportional to the heat produced with each injection. The lower panel shows the integrated areas normalized to the number of moles of apomethylmalonyl-CoA mutase injected at each injection step. The data were fitted to a single-binding site model, and the resulting thermodynamic parameters for complex formation are listed in Table 2. B, Temperature dependence of the enthalpy change for binding of MeaB to methylmalonyl-CoA mutase. The values (± S.D.) observed for the binding enthalpy change during complex formation between the apomutase and MeaB-GMPPNP (○) or MeaB-GDP (■) and between the holomutase and MeaB-GMPPNP (△) or MeaB-GDP (●) are plotted versus temperature. The slopes of the plots, obtained from the best fit lines shown, yielded the binding heat capacity changes (ΔC°) in Table 2. C, calorimetric data for a representative titration of GDP (160 μM) and apomethylmalonyl-CoA mutase-MeaB (11.5 μM) in buffer B lacking KCl. The resulting thermodynamic parameters for the effect of methylmalonyl-CoA mutase on binding of effectors are listed in Table 3.

**TABLE 2**
Thermodynamic parameters for binding of methylmalonyl-CoA mutase to MeaB-nucleotide complexes

<table>
<thead>
<tr>
<th>MCM</th>
<th>MeaB</th>
<th>ΔΗ°</th>
<th>TΔS°</th>
<th>ΔG°</th>
<th>Kd (μM)</th>
<th>ΔC°</th>
<th>ΔH°</th>
<th>ΔS°</th>
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<td>-24.1 ± 0.5</td>
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<td>-27.2 ± 1.2</td>
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<td>-416</td>
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<td>2046</td>
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Energetics of MeaB-MCM Interaction

<table>
<thead>
<tr>
<th>TABLE 3</th>
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<tr>
<td>Effect of methylmalonyl-CoA mutase on the energetics of nucleotide binding to MeaB at 20 °C</td>
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<tr>
<td>These titrations were performed in buffer B lacking KCl. The thermodynamic parameters are the averages of two experiments (± S.D.).</td>
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<table>
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<tr>
<th>MCM</th>
<th>Nucleotides</th>
<th>$K_d$</th>
<th>$\Delta H^\circ$</th>
<th>$T \Delta S$</th>
<th>$\Delta G^\circ$</th>
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<tr>
<td>None</td>
<td>GDP</td>
<td>1.3 ± 0.1</td>
<td>$-13.6 \pm 0.5$</td>
<td>$-5.7 \pm 0.4$</td>
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<td></td>
<td>GMPPPNP</td>
<td>5.6 ± 0.4</td>
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<td></td>
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fold tighter than of apomutase and is driven by a substantial decrease in the enthalpy of association (Table 2). The change in the binding heat capacity for formation of the holomutase-MeaB-GMPPNP and holomutase-MeaB-GDP complexes are $-865$ and $-416$ cal/(mol K), respectively, and correspond to $~6950$ and $~4000$ Å$^2$ of surface area becoming buried. From the experimental enthalpy of association, it is estimated that 71 and 35 residues become ordered as holomutase binds to MeaB-GMPPNP and MeaB-GDP, respectively. Binding of holomutase to MeaB containing GDP instead of GMPPNP is less favorable by a factor of 15. This change in the dissociation constant is largely governed by a smaller decrease in $\Delta H^\circ$.

Effect of Methylmalonyl-CoA Mutase on Binding of Nucleotides to MeaB—Having characterized the effect of nucleotide-bound MeaB on association with methylmalonyl-CoA mutase, we next examined the effect of the mutase on binding of nucleotides by MeaB (Fig. 3C and Table 3). The addition of apomutase decreased the $K_d$ for GDP and GMPPNP binding to MeaB by factors of 6.5 and 51, respectively. The presence of holomutase has essentially no effect on the affinity of MeaB for GDP and decreased the $K_d$ for GMPPNP by $\sim 8$-fold.

Effect of Methylmalonyl-CoA Mutase on the GTPase Activity of MeaB—As predicted for a P-loop protein with an aspartate in the magnesium binding site, MeaB binds and hydrolyzes GTP, although the kinetics of this reaction have not been reported (7). The kinetics of GTP hydrolysis catalyzed by MeaB was characterized by examining the time-dependent increase in GDP at various initial concentrations of GTP (Fig. 4). A Michaelis-Menten analysis of the data yielded the following parameters: $K_{m(GTP)} = 150 \pm 20$ µM and $k_{cat} = 0.039 \pm 0.003$ min$^{-1}$ at 37 °C. The addition of apo-methylmalonyl-CoA mutase stimulated the GTPase activity of MeaB 108-fold to 4.2 ± 0.2 min$^{-1}$ (Fig. 5). The addition of holo-methylmalonyl-CoA mutase increased the GTPase activity of MeaB by 123-fold, corresponding to $k_{cat} = 4.8 \pm 0.2$ min$^{-1}$ (Fig. 5).

FIGURE 4. GTPase activity of MeaB. A, kinetics of GTP hydrolysis. The reactions were performed by incubating 10 µM MeaB with varying concentrations of GTP (50–5000 µM) in 50 mM HEPES, pH 8, 300 mM KCl, 10 mM MgCl$_2$, 5% glycerol at 37 °C and monitoring the time-dependent increase in GDP formation as described under “Experimental Procedures.” B, Michaelis-Menten analysis of the rate of GTP hydrolysis by MeaB at varying concentrations of GTP yielded a correlation coefficient, $r^2 = 0.98$, $k_{cat} = 0.039 \pm 0.003$ min$^{-1}$, and $K_m(GTP) = 150 \pm 20$ µM.

FIGURE 5. Effect of methylmalonyl-CoA mutase on the GTPase activity of MeaB. The reactions were performed by incubating 10 µM apo- or holo-methylmalonyl-CoA mutase (circle), MeaB (triangle), apo-methylmalonyl-CoA mutase-MeaB (square), or holo-methylmalonyl-CoA mutase-MeaB (diamond) with 5 mM GTP in 50 mM HEPES, pH 8, 300 mM KCl, 10 mM MgCl$_2$, 5% glycerol at 37 °C and monitoring the time-dependent increase in GDP formation as described under “Experimental Procedures.” The GTPase activities of MeaB alone, in complex with apo- or holo-methylmalonyl-CoA mutase were 0.039 ± 0.000, 4.2 ± 0.2, and 4.8 ± 0.2 min$^{-1}$, respectively.

DISCUSSION

As a prototype of the family of G-protein cofactor chaperones, we have chosen to characterize MeaB and its target protein, methylmalonyl-CoA mutase, from M. extorquens AM1. The predicted molecular mass of the polypeptide is $\sim 35$ kDa, and it has been reported to exist in solution as a monomer (7). However, gel filtration chromatography and dynamic light scattering studies revealed that MeaB is a dimer and thus resembles homologs, viz. UreG (4), HypB (20), and CooC (6), which are reported to be homodimers.

MeaB forms a stable complex with methylmalonyl-CoA mutase, which can be separated on a native gel. A binding stoichiometry of $\sim 1:1$ for dimeric MeaB-heterodimeric methylmalonyl-CoA mutase was seen
when formation of the complex was titrated with increasing concentrations of MeaB-GMPPNP (Fig. 2).

The differential effects of GDP versus GMPPNP and apo- versus holo-mutase on the thermodynamics of interaction with MeaB were revealed by titration calorimetry (Tables 1–3). MeaB exhibits similar affinities for GMPPNP, a nonhydrolyzable analog of GTP and GDP ($K_d$ of 7.3 ± 0.6 and 6.2 ± 0.7 μM, respectively, in the presence of 300 mM KCl at 20 °C) and thus belongs to the subclass of GTPases characterized by low nucleotide affinity (21). Other examples of GTPases that similarly non-discriminatory in their binding preference for GTP versus GDP include members of a family of interferon-inducible GTPases, viz. Mx and hGBP1. The presence of the mutase enhances the affinity for the nucleotides with the strongest effect being observed for GMPPNP binding to the apomutase (51-fold increase) or to the holomutase (8-fold increase) (Table 3).

The dissociation constant for binding of methylmalonyl-CoA mutase and MeaB ranges from 34 to 524 nM at 20 °C depending on the combination of nucleotide and the form of mutase employed (Table 2). Interestingly, although holomutase exhibits a 15-fold higher affinity for the GMPPNP-bound form of MeaB versus the GDP-bound form, the apomutase does not show a similar preference, binding both forms with comparable affinity.

The extent of structural reorganization that accompanies complex formation can be assessed from relationships that correlate binding and folding energetics with structure (22, 23). The $\Delta C^\circ$ values yield estimates of the surface area that becomes buried during complex formation. Binding of apo- and holo-mutase to a complex of MeaB-GMPPNP is estimated to bury 8600 and 5360 Å² of surface area, respectively. Although a three-dimensional structure of the complex of these two proteins is not available, based on the magnitudes of the nonpolar surfaces that become buried, they are unlikely to be restricted to the interface. Structural rearrangements distal to the protein-protein interface are also predicted from a similar analysis for the interaction between the CD4 receptor and gp120, the external envelope glycoprotein of the HIV (17). Indeed, because the magnitudes of the nonpolar surfaces that become buried in site specific complexation of macromolecules is often too large to be ascribed to “rigid body association,” it has been postulated that they reflect conformational changes that are coupled to binding (24, 25).

The presence of a fully conserved P-loop motif in MeaB predicts that it has GTPase activity. MeaB has modest intrinsic GTPase activity ($k_{cat}$ of ~0.04 min$^{-1}$ at 37 °C), which is comparable with that of UreG (0.04 min$^{-1}$ (4)) and slightly lower than of HypB (~0.17 min$^{-1}$ (5)) and CooC (0.23 min$^{-1}$ (6)). The presence of apo- or holo-mutase has a significant effect, enhancing the GTPase activity by 2 orders of magnitude. These results suggest that the mutase functions as a mild GAP (guanine nucleotide-activating protein) and that in its presence, MeaB would accumu-
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late in the GDP-bound state, which has a lower affinity for the MeaB-mutase complex (Scheme 1).

A variety of processes in nature are regulated by guanine nucleotide-binding proteins that act as molecular switches that toggle between inactive GDP-bound and active GTP-bound states. A conserved G-domain fold is common to members of this superfamily and harbors the essential motifs for the switch (16). A homology model of MeaB was generated using the coordinates for the crystal structure for the signal sequence-binding protein from *Thermus aquaticus* (Fig. 6). The predicted structure has a core α/β G domain comprised of parallel β-strands surrounded by α-helices. An N-terminal Walker A motif contains a GXXGXGK(S/T) sequence that is predicted to function in positioning the triphosphate moiety of the bound nucleotide. The Walker B motif contains a conserved aspartate at its N-terminal end that may play a structural role, and the (N/T)KXD sequence confers specificity for guanine (2). The N- and C-terminal regions of MeaB appear to be organized into discrete modules in the modeled structure, and one or both could be involved in interactions with methylmalonyl-CoA mutase and/or dimer formation.

In summary, elucidation of the thermodynamics of interaction between MeaB with methylmalonyl-CoA mutase provides insights into their modulation by nucleotides, which in turn affects the intrinsic GTPase activity of MeaB as shown in Scheme 1. MeaB binds GMPPNP ~4-fold more tightly than GDP, although this difference disappears when the titrations are conducted in the presence of 300 mM KCl (Table 1), which is likely to be more physiologically relevant. Methylmalonyl-CoA mutase binds to the MeaB-GTP complex and stimulates the GTPase activity of MeaB by a factor of ~100. The resulting MeaB-GDP form has a 15-fold lower affinity for the mutase. In contrast, apo-mutase does not discriminate between the GDP- and GMPPNP-bound forms of MeaB in terms of binding affinity. These data suggest that the enhanced GTPase activity in the MeaB-mutase complex could be harnessed in the auxiliary role that MeaB plays in the mutase-catalyzed reaction in the cell and as shown in the model in Fig. 1.

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