Crystal Structure and Mutagenesis of the Metallochaperone MeaB

INSIGHT INTO THE CAUSES OF METHYLMALONIC ACIDURIA

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MeaB is an auxiliary protein that plays a crucial role in the protection and assembly of the B 12 -dependent enzyme methylmalonyl-CoA mutase. Impairments in the human homologue of MeaB, MMAA, lead to methylmalonic aciduria, an inborn error of metabolism. To explore the role of this metallochaperone, its structure was solved in the nucleotide-free form, as well as in the presence of product, GDP. MeaB is a homodimer, with each subunit containing a central α/β-core G domain that is typical of the GTPase family, as well as α-helical extensions at the N and C termini that are not found in other metalloenzyme chaperone GTPases. The C-terminal extension appears to be essential for nucleotide-independent dimerization, and the N-terminal region is implicated in protein-protein interaction with its partner protein, methylmalonyl-CoA mutase. The structure of MeaB confirms that it is a member of the G3E family of P-loop GTPases, which contains other putative metallochaperones HpyB, CooC, and UreG. Interestingly, the so-called switch regions, responsible for signal transduction following GTP hydrolysis, are found at the dimer interface of MeaB instead of being positioned at the surface of the protein where its partner protein methylmalonyl-CoA mutase should bind. This observation suggests a large conformation change of MeaB must occur between the GDP- and GTP-bound forms of this protein. Because of their high sequence homology, the missense mutations in MMAA that result in methylmalonic aciduria have been mapped onto MeaB and, in conjunction with mutagenesis data, provide possible explanations for the pathology of this disease.

In the past few decades, an increasing number of guanine nucleotide-binding protein (G proteins) that act as chaperones in the assembly of target metalloenzymes have been described (1). These include UreG (2, 3), HpyB (4, 5), CooC (6), and MeaB (7), which are involved in the metallocenter assembly of urease, NiFe-hydrogenase, CO dehydrogenase, and B 12 -dependent methylmalonyl-CoA mutase, respectively. Typically, G proteins act as molecular switches, with regions known as switch I and switch II undergoing large conformational changes upon GTP hydrolysis to communicate a signal. Although members of this metallochaperone G protein subfamily (called the G3E family) share appropriate sequence motifs (8) and exhibit low GTPase activity (3, 4, 6, 9), their exact function with respect to target metalloenzymes remains to be determined. MeaB itself differs from the other G3E G proteins in that it possesses N- and C-terminal extensions of unknown function.

MeaB is an auxiliary protein associated with methylmalonyl-CoA mutase (MCM) 2 (7, 9, 10), a coenzyme B 12 (adenosylcobalamin)-dependent enzyme that catalyzes the chemically challenging 1,2-rearrangement of methylmalonyl-CoA to succinyl-CoA using radical-based chemistry (11, 12). A human orthologue of MeaB, MMAA, has been found to be the locus of mutations associated with type A (cblA) methylmalonic aciduria (MMA) (13), a rare congenital disease that manifests itself during fetal development or shortly after birth; symptoms include chronic acidosis and mental retardation (13). This genetic evidence identifies MeaB/MMAA as an important auxiliary protein for MCM. While its exact role is currently being investigated, MCM requirement for coenzyme B 12 suggests several possibilities for MeaB function, including roles in protecting the enzyme from oxidative inactivation and cofactor insertion/removal (7, 9, 10).

Naturally occurring forms of B 12 are powerful catalysts; used for radical-generation in the case of adenosylcobalamin and as potent nucleophiles for methyl transfer reactions in the case of cob(1)alamin. Because of their highly reactive nature, the cobalamin cofactor can be easily inactivated, inhibiting the enzyme.

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The atomic coordinates and structure factors (code 2qm7 and 2qm8) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: MCM, methylmalonyl-CoA mutase; MMA, methylmalonic aciduria; MMAA, methylmalonic aciduria type A; ATR, ATP:cobalamin adenosyltransferase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PEG, polyethylene glycol; MAD, multiple wavelength anomalous dispersion; NCS, non-crystallographic symmetry; TCEP, Tris(2-carboxyethyl)phosphine; r.m.s., root mean square; GTPγS, guanosine 5′-O-(thiotriphosphate); GMP-PNP, guanosine 5′-(β,γ-imino)triphosphate.
Because this inactivation is highly undesirable, nature has developed at least three possible remedies, all of which involve accessory proteins: (i) reduction in situ of a B\textsubscript{12} intermediate that has undergone oxidative inactivation, (ii) replacement of the inactive B\textsubscript{12} cofactor with a new cofactor, and (iii) protection of the reactive B\textsubscript{12} species by sequestration from solvent. Methionine synthase, the only other B\textsubscript{12}-dependent enzyme in mammals, uses NADPH-dependent methionine synthase reductase to reductively reactivate the oxidized inactive B\textsubscript{12} cofactor (14). A corresponding reductase for MCM has not been found. B\textsubscript{12}-dependent eliminases like diol dehydratase use an ATP-dependent mechanism to exchange the oxidized inactive cofactor for an active one (15); however, such chaperones do not appear to be present in operons that encode MCM. Thus MCM does not appear to utilize the first two methods, leaving the third possibility that MeaB could play a role in cofactor protection during catalysis. This hypothesis has been investigated by testing the rate of cob(II)alamin oxidation on MCM in the presence of various nucleotide-bound forms of MeaB (10). Results indicate that GTP-MeaB affords 15-fold protection in a GTPase-independent manner, suggesting that protection from oxidative damage during catalysis is one role for this chaperone. It is interesting that the protection afforded by GDP-MeaB is much less (3-fold) than for GTP-MeaB, as is the estimated buried surface area between MeaB and MCM (~4000 \AA\textsuperscript{2} for GDP vs 6950 \AA\textsuperscript{2} for the GTP form), indicating that the structure of GTP-MeaB and the structure of GDP-MeaB are notably different. In thinking about how GTP-MeaB could protect the cobalamin cofactor in a GTPase-independent fashion, one must consider that crystallographic studies show that MCM undergoes a major conformational change during catalysis; a substrate-binding TIM barrel splits open allowing product dissociation, and seals closed again upon substrate binding (16). During these conformational changes, the simple binding of MeaB to MCM could help to sequester the MCM-bound cofactor from solvent. Because many metallochaperones have been implicated in cofactor insertion (17–19), it is also important to consider this function for MeaB. For MCM, it is postulated that ATP:adenosylcobalamin adenosyltransferase (ATR) delivers the adenosylcobalamin cofactor to MCM; however, for MCM to accept this cofactor, crystallographic analysis suggests that MCM would have to undergo a large conformational change that repositions the B\textsubscript{12}-binding Rossmann domain away from the substrate-binding TIM barrel (16). Here, the GTPase-dependent activity of MeaB could play a role in reconfiguring the MCM structure to accept the cofactor from ATR. Thus, while the detailed functions of MeaB are still being established, potential roles in cofactor assembly and protection are intriguing possibilities to consider, and there is no doubt as to the importance of MeaB proteins in MCM activity based on cblA MMA disease data.

Because of their inherent propensity to unfold and aggregate, to date only two G3E GTPases have been structurally characterized (5, 36). While sequence homology with other GTPases strongly suggests the G domain folds in a manner similar to other structurally known GTPases, the significance of the N- and C-terminal extensions found in MeaB are unknown. To investigate the significance of these amino acid extensions, as well as to gain an understanding of the structural basis of MMAA and the role MeaB plays in regulating MCM activity, we determined product-bound and substrate-free structures of MeaB from Methylobacterium extorquens AM1. Our structural results suggest that the C terminus is critical in formation of the homodimer, while we speculate that the N terminus may function in the interaction with MCM. In addition, mapping the point mutations known to correlate with the occurrence of MMAA onto the structure of MeaB, in conjunction with biochemical analysis of these mutants, allows us to propose the structural basis for this disease. Finally, the overall topology of this enzyme gives clues as to how MeaB associates with MCM to influence mutase activity.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—MeaB from *M. extorquens* was expressed using the pET21d::meaB vector (generously provided by Dr. Mary Lidstrom, University of Washington) and purified as described previously (9). Mutants were made using the QuikChange site-directed mutagenesis kit (Stratagene). The following sense mutagenic primers were used: L111P (5′-CGACATGGACACCCCCGAGCGCCG-3′), R57Q (5′-ACGGGGCGGGCAGTCAGTTTGCC-3′), F119C (5′-CTCGCCATCGACCCGAAACCCGTCA-3′), R272Q (5′-GACCCGCGAGATCGCGGGGCAAGCAGGCGAGACGTGAAATG-3′). The presence of the mutation was verified by DNA sequencing. All mutants were purified in the same manner as wild type MeaB. For crystallization, purified native protein was concentrated to ~7 mg/ml in 50 mM HEPES, pH 8.0, 300 mM KC\textsubscript{1}, 2.5 mM MgCl\textsubscript{2}, and 10% glycerol, and stored at ~80 °C prior to use. Selenomethionine-labeled (SeMet) MeaB protein was expressed in an identical manner, but with cells grown in minimal media supplemented with l-SeMet and concentrated to ~8.4 mg/ml.

**Methylmalonyl-CoA Mutase Assay**—The specific activity of methylmalonyl-CoA mutase was analyzed using the radiolabel-based assay at 37 °C as previously described (20). The effect of the MeaB F119C mutant on the kinetic parameters of methylmalonyl-CoA mutase was determined in the presence of 1 mM Mg-GDP, 50 \mu M 5′-deoxyadenosylcobalamin, and 2.5 mM (R,S)-[14C]methylmalonyl-CoA in 50 mM potassium phosphate, pH 7.5, by varying the concentration of MeaB F119C from 2 to 75 nm.

**Quantification of Free Thiol Groups**—The free thiol group content of wild type MeaB and the F119C mutant (15 \mu M) was determined by titration with 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma) in 50 mM HEPES, pH 8.0, containing 300 mM NaCl. After incubation for 15 min at room temperature, the absorbance at 412 nm was recorded, and the number of free thiol groups was calculated using an extinction coefficient of 13,600 M\textsuperscript{-1} cm\textsuperscript{-1} (21).

**Protein Crystallization**—Crystals of the native form of MeaB with GDP bound were grown at room temperature using the hanging drop technique; 1.5 \mu l of native protein at ~7 mg/ml in 50 mM HEPES, pH 8.0, 300 mM KCl, 2.5 mM MgCl\textsubscript{2}, and 10% glycerol was added to 1.0 \mu l of precipitant solution (0.1 M Bis-Tris, pH 5.5, 0.2 M Li\textsubscript{2}SO\textsubscript{4}, and 17% polyethylene glycol (PEG)
Crystal Structure and Mutagenesis of MeaB

TABLE 1
Data collection statistics

|                       | SeMet GDP-MeaB | GDP-MeaB | Apo-(SeMet) MeaB |
|-----------------------|----------------|----------|------------------|
| Wavelength (Å)        | 0.9791         | 0.9252   | 0.9794           |
| Resol. (Å)            | 30.0-2.0       | 30.0-2.0 | 30.0-1.70        |
| Completens            | 99.9 (99.9)    | 98.6 (98.9) | 98.5 (89.6)    |
| Mosaicity (°)         | 0.523          | 0.454    | 0.490            |

Merged using SCALEIT

Phasing resol. (Å)   30.0-2.8
# Sites             12
Phasing power (iso./anom.) 1.2/1.2
FOM (acent./cent.)  0.72/0.59
Phase extension to 2.0 Å with SOLOMON

*Highest resolution shell = 2.07-2.0 Å.
*Highest resolution shell = 1.92-1.85 Å.
*Highest resolution shell = 1.76-1.70 Å.

3350) and placed over 0.5 ml of precipitant. In addition, 0.2 µl of GDP dissolved in water was added to the drop to give a final nucleotide concentration of 2 mM in the crystallization reagent, while 0.2 µl of 0.4 mM Zwittergent 3-14 was also included to reduce layering of crystals. Cryoprotection of native crystals was achieved by immersion in precipitant solution containing 20% ethylene glycol, before being flash frozen in liquid nitrogen. SeMet MeaB was crystallized with GDP using the same precipitant solution, but with ~20% (v/v) ethylene glycol used in place of Zwittergent 3-14.

After optimization, crystals could be grown that generally appeared single, having maximum dimensions of 400 × 400 × 50 µm after 3–4 days. Unfortunately, even though these crystals appeared single, upon examination of diffraction properties, the majority contained multiple crystal lattices. As a result, many crystals were tested until one deemed suitable for data collection was found.

In an attempt to determine the structure of MeaB bound with a GTP analogue, SeMet MeaB was crystallized under identical conditions to the SeMet MeaB:GDP complex, but with 10 mM non-hydrolyzable GTP analog GMP-PNP in 160 mM MgSO₄ used in place of GDP. The selenomethionine-labeled protein was used because of its superior crystallization and diffraction qualities over native MeaB, and the fact that it is perfectly isomorphous to the native form. Analysis of the initial Fc − Fo map showed no nucleotide was bound, which was subsequently confirmed during refinement. This crystal form was therefore considered to be the nucleotide-free, or apo-form.

Data Processing, Model Building, and Refinement—Multiple Wavelength Anomalous Dispersion (MAD) data on a single SeMet-GDP MeaB crystal were collected using the inverse beam technique around the selenium absorption edge at beam line 11-1, Stanford Synchrotron Radiation Laboratory (SSRL). Data on a GDP-MeaB crystal were collected to 1.85 Å at the Argonne National Laboratory Advanced Photon Source (APS), beam line 17-ID. High resolution diffraction data of the apo-form were collected at beam line 9-2, SSRL. Data were integrated and scaled using HKL or HKL-2000, with the MAD data scaled together using SCALEIT (23), part of the CCP4 suite of programs (24). Selenium sites were located automatically using SOLVE (25), and were subsequently refined and phased in SHARP (26) using all MAD data. Data collection statistics are summarized in Table 1. About 70% of the model, containing two molecules per asymmetric unit, was automatically built in RESOLVE using Non-Crystallographic Symmetry (NCS) averaging (25), and was completed and refined using alternating model building in Coot (27) and reciprocal space refinement in CNS (28), without NCS restraints and using a sigma cutoff of 0.0. Experimental and model phases were combined at each step using SigmaA (29) to reduce model bias.

During refinement, three unexpected point mutations were discovered; Leu²⁹² → Phe, Arg²⁴ → His, and Gly³⁵⁷ → Asp. Sequencing of the original plasmid construct (7) confirmed all three point mutations; however, these mutations do not appear to affect the overall properties of this enzyme.³ None of these loci appear to form any crystal contacts, and there are no interactions within the asymmetric unit that appear to suggest this triple mutant confers any advantage in crystallographic studies over wild type.

Once the GDP-bound SeMet model was complete, the B-factors were reset, and the model refined against the high resolution GDP-MeaB data. Water molecules were then included automatically using ARP/wARP (30), with additional reciprocal space refinement in REFMAC (31). Finally, manual addition and deletion of water molecules was performed until R_free convergence was achieved. The final model includes residues 5 to 98 and 108 to 327 out of 329 residues of molecule 1, and resi-

³ D. Padovani and R. Banerjee, unpublished observations.
TABLE 2
Model statistics

|                | Crystal | GDP-MeaB | Apo-MeaB |
|----------------|---------|----------|----------|
| Resol. (Å)     | 30.0-1.85 | 30.0-1.70 |          |
| Rcryst/Rfree (%) | 19.6/24.7 | 17.5/20.8 |          |
| Average B-factors (Å²) |          |          |          |
| Protein [no. atoms] | 26.1 [4673] | 19.6 [4766] |          |
| GDP [no. atoms] | 21.1 [56] | – |          |
| Solvent [no. atoms] | 36.5 [454] | 34.8 [872] |          |
| Others* [no. atoms] | 30.8 [10] | 32.1 [20] |          |
| r.m.s. deviation |          |          |          |
| Bond lengths (Å) | 0.015 | 0.011 |          |
| Bond angles (°) | 1.5 | 1.2 |          |
| Ramachandran analysis |          |          |          |
| Most favored (%) | 97.4 | 96.1 |          |
| All additional allowed (%) | 2.6 | 3.9 |          |

* Buffer molecules, phosphate.

The crystals of apo-MeaB, derived from an unsuccessful attempt to co-crystallize MeaB with the GTP analog GMP-PNP, were formed under the same conditions as the GDP-bound form, also belonging to space group P2₁; however, the crystals have different unit cell parameters (see Table 1). Therefore, the GDP-bound model was used as a probe for molecular replacement using PHASER (32), with rigid-body refinement confirming the solution to be correct. The model was then refined using the same protocol for the GDP-bound structure, with the same FreeR flags used in both datasets. The refined apo model includes residues 4–180 and 187–329 of molecule 1, and residues 6–203 and residues 210–329 of molecule 2. Model refinement statistics are listed in Table 2.

RESULTS

Overall Structure of MeaB—Inspection of the crystal lattice shows two molecules of MeaB per asymmetric unit, with their arrangement suggesting the protein to be a homodimer, consistent with results obtained from gel filtration and light scattering experiments (9). Each monomer can be considered as being composed of three regions; a central G domain, which includes a 7 parallel-stranded β-sheet that houses the GT Pase active site, and α-helical extensions at both the N and C termini (Fig. 1). The C-terminal extension, composed of ~70 amino acids, appears critical in forming the dimer interface as its three α-helices interlock with the neighboring subunit (highlighted in Fig. 1). However, the total intermolecular interface is relatively small, being just 4800 Å², or about 18% of the total molecular surface. The N-terminal extension is also composed of three α-helices, totaling ~50 amino acids. This region resides near the C-terminal dimerization extension, but on the opposite side of the molecule to the active site.

The G Domain—Inspection of the G domain shows it possesses the same topology as other structurally characterized members of the SIMIBI class, being composed of a 7-stranded parallel β-sheet embellished by α-helices. SIMIBI and TRAFAC are the two major classes of G proteins (8), and while TRAFAC G proteins display a six-stranded β-sheet containing one antiparallel strand, SIMIBI G proteins are recognized by the presence of a seven-stranded parallel β-sheet. In comparison of MeaB with one of the better studied SIMIBI G proteins, Ffh (Protein Data Bank code 1R9) (33), the r.m.s. deviation of 113 Cα-atoms is only 1.8 Å. Interestingly, the structural homology is not quite as good when MeaB is compared with HypB (Protein Data Bank code 2HF9) (5), which unlike Ffh belongs in the same G3E subfamily of GT Pases (the metallochaperone subfamily). For 91 Cα atoms (as opposed to 113), the r.m.s. deviation between MeaB and HypB is 1.9 Å (compared with 1.8 Å for Ffh).

GT Pases are characterized by four motifs: (i) the P-loop (Walker A), which coordinates the phosphate moieties of the guanine nucleotide through hydrogen bonding with backbone nitrogen atoms, (ii) a guanine base specificity loop, (iii and iv) switch I and II regions that interact with the nucleotide and Mg²⁺ in the active site, activate water for GTP hydrolysis, and signal via conformational change that GTP hydrolysis has occurred. While functions of these motifs are conserved among the various subfamilies of GT Pases, there are sequence differences that serve to identify proteins as particular subfamily members. Structure analysis of GDP-MeaB confirms that it is a member of the G3E (metallochaperone) family, containing the appropriate sequence motifs at the expected places in the structure (8). For the P-loop (Walker A motif) we find the expected GXXGXXGK(S/T) variant in the form of 62GVPGVGKS99 providing backbone amide nitrogens to interact with the phosphate moieties of GDP (Figs. 1, 2, and 3), and for the base specificity loop, we find the expected intact NKXD motif in the form of 206NKAD209 (Figs. 1–3). In the switch II (or Walker B) region, G3E proteins substitute a glutamate for the conserved Mg²⁺-binding-aspartate residue (8). Although Mg²⁺ is not bound in any of our structures, the switch II motif (Glu¹⁵⁴,Gly¹⁵⁷) houses Glu¹⁵⁴ which, according to structural alignment with HypB Glu¹₂₀, is likely to coordinate Mg²⁺ in the GTP-bound enzyme form. In addition to Glu¹⁵⁴, Ser⁶⁹ from the P-loop of MeaB is likely to coordinate the Mg²⁺ ion (homologous to Thr⁴⁷ in HypB). HypB shows a third amino acid, Asp⁷⁵, involved in direct coordination with Mg²⁺, and forms part of an α-helix (Fig. 2). The corresponding region in the GDP-bound form of
MeaB is completely disordered, and is a random coil in the apo-form; however, it is possible that when bound to GTP, the corresponding residue, Asp^{105}, may reposition itself to coordinate the metal ion. By structural comparison with the GTPγS-bound structure of HypB, Asp^{92} appears to be the most likely candidate for part of the switch I region sequence motif, responsible for positioning a water molecule for in-line nucleophilic attack of the γ-phosphate, as well as for communicating GTP hydrolysis.

**Comparison of MeaB with HypB**—The recently published crystal structure of HypB (5) shows that although MeaB and HypB share similar G domain topologies (Fig. 4A), the assembly of the homodimer is significantly different between the two enzymes (Fig. 4B). For HypB, the nucleotide binds at the HypB dimer such that the GTPase active site is formed by both subunits (Figs. 2 and 4). This domain arrangement is in stark contrast to MeaB, in which the active sites are situated on the periphery of each subunit (Fig. 4). Furthermore, dimerization of HypB is nucleotide dependent (5), whereas MeaB is not (9). The superposition of MeaB and HypB G domains (Fig. 4) shows that the G domain fold is extremely similar (r.m.s. deviation of 1.9 Å) and not the cause of this difference in dimerization or active site location. Instead, the C-terminal extension, which is unique to MeaB in the G3E family, appears to be the responsible factor. This C-terminal region from one monomer is intertwined with the other, creating a novel dimer interface for this G3E family member. It is also important to note that in the GDP-bound form of MeaB, the switch I and II regions, which should communicate GTP hydrolysis, are pointed away from any protein surface that is likely to interact with MCM (Figs. 1 and 7). It is unclear how positioning of the switch I and II regions at the center of the dimer could effectively communicate a signal, suggesting that the GTP-bound form of MeaB is likely to have a different conformation, in agreement with biochemical data (9). It will be interesting to compare the structures of GTP- and GDP-bound forms of MeaB to see if and how this domain interface changes. In contrast to the C-terminal extension, the N-terminal extension plays no role in dimerization.
because of its projection away from the G domain core, we predict that this region of protein, unique to MeaB, is involved in interaction with MCM.

Mapping Point Mutations of MMAA onto MeaB—If one excludes the putative N-terminal mitochondrial targeting sequence of MMAA, MeaB, and MMAA share 46% sequence identity and 67% homology over the entire length of each peptide (Fig. 3). This sequence similarity suggests the fold of MMAA bears a strong resemblance to MeaB, making it possible to map point mutations known to cause MMA in the cblA complementation group (34) onto the crystal structure of MeaB (Fig. 5).

Leu11 (MeaB numbering, Leu89 in MMAA) is located in the N-terminal α-helix. Mutation of this amino acid to proline, which presumably results in destruction of this α-helix, is known to result in MMA. This α-helix does not appear to participate in dimer formation, or form part of the catalytic G domain; however, it may be part of the interface that contacts MCM. Therefore, mutation in this region in the human homolog, MMAA, may compromise MCM function through its inability to interact with MCM, and thus bring about MMA. Attempts to express this mutant in _Escherichia coli_ suggest that stability may also be an issue, as this protein aggregates and accumulates in inclusion bodies (data not shown).

The next two amino acids whose mutations are known to cause MMA, Arg57 and Arg272 (MeaB numbering, 145 and 359 in MMAA), reside next to each other in a highly charged region of the G domain that also includes Arg137 and Arg259 (Fig. 6A). This rather unusual arrangement of 4 arginine residues, which are charge-neutralized by forming salt bridges with nearby acidic amino acids, may be critical in stabilizing interactions between the G domain and the C-terminal dimerization region, and might explain why mutation of either residue to glutamine is pathogenic (34). Expression of the R57Q and R272Q proteins appear to confirm this as both mutants are expressed in inclusion bodies (data not shown).

The inspection of Phe119 in MeaB, which in MMAA is replaced with the paralogue Tyr209, shows the C1 atom is ~5 Å from the sulfur atom of Cys143, corresponding to Cys233 in MMAA (Fig. 6B). Because the Y209C mutation associated with MMA would place two thiol groups in such close proximity, the redox environment of the mitochondrion may promote disulfide bond formation. As with the other mutant proteins characterized,
stability of the F119C mutant is an issue. However, the protein can be kept soluble in the presence of 5 mM DTT, though experiments had to be conducted within a day of purification. Like wild type MeaB, the F119C mutant activated methylmalonyl-CoA mutase 2-fold in the standard in vitro assay (data not shown). To test the hypothesis that conversion of Phe119 to a cysteine could result in a disulfide bond with Cys143, thiol titrations were performed. Wild-type enzyme has a single cysteine (Cys143), and DTNB titration revealed the presence of two free thiol groups per dimer. In contrast, the F119C mutant, which has two cysteines (Cys143 and Cys119) revealed the presence of two free thiols in the protein as isolated and four thiols when the protein was pretreated with a reductant, 5 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP, Pierce) (Table 3). This result is consistent with the presence of a disulfide bond in one subunit and two free thiols in the other.

The next two mutations involve substitution of glycine residues, Gly147 and Gly218 (corresponding to Gly59 and Gly130 in MeaB), with glutamate. The first residue, Gly59, is tightly packed into the core of the G domain (Fig. 5). Mutation to a relatively bulky amino acid such as glutamic acid might result in steric and electrostatic clashes that destabilize the core of the protein and cause it to fold improperly. The second residue, Gly130, resides on the surface of the protein near the inter-subunit interface (Fig. 5). As such, it is possible that the hydrophobic to hydrophilic G218E mutation may adversely affect assembly of MAAA, with the consequence that it is no longer able to interact with MCM. The more restricted conformational flexibility created by a glycine to glutamate mutation, on the other hand, is unlikely to be a cause of disease since position 130 (MeaB numbering) is in an α-helix. If anything, Glu would be more favorable than Gly in terms of helical propensity.

Comparison of the Substrate-free and -bound Forms—Efforts to crystallize MeaB with a GTP substrate analog, either GMP-PNP or GDP with aluminum fluoride, have so far proved fruitless. Structure determination of MeaB crystals soaked or grown in the presence of excess GMP-PNP results in an apo- (substrate-free) form. Although the unit cells of the GDP-bound and apo-forms are different (Table 1), requiring initial phases to be calculated through molecular replacement, the crystallization conditions and space groups are the same. Furthermore, the overall folds of the two states are nearly identical; having an r.m.s. deviation of 0.8 Å for Ca atoms (illustrated as a “sau-

| Protein               | SH groups/dimer | No. disulfide bonds/dimer |
|-----------------------|-----------------|---------------------------|
| Wild-type MeaB        | 2.0 ± 0.07      | 0                         |
| F119C MeaB            | 2.2 ± 0.15      | 1                         |
| F119C MeaB + TCEP     | 4.2 ± 0.09      | 0                         |

FIGURE 6. Two locations of MMA-associated mutations. A, ball-and-stick diagram outlining the unusual arrangement of four arginines (yellow), including Arg57 and Arg272, which are stacked against each other. Both side chains are covered with a 2 F0 – Fc map in green mesh, contoured at 1σ. Acidic residues which counter-balance the positively charged environment are in red. B, relatively short distance between the C1 atom of Phe119 and the thiol group of Cys143 is highlighted as a dotted black line.

FIGURE 7. Differences in Ca positions between the GDP-bound and apo-forms of MeaB (thicker lines indicate larger conformational changes). Positions of the switch regions (those parts of the protein that “communicate” GTP hydrolysis to a partner protein) are annotated. The black dotted circle highlights a conformational change near the nucleotide binding site at the dimer interface.

Crystal Structure and Mutagenesis of MeaB
Crystal Structure and Mutagenesis of MeaB

MeaB is a homodimeric GTPase found throughout all forms of life, with the human homologue, MMAA, facilitating MCM in the catabolism of cholesterol, branched chained amino acids and odd chain fatty acids (35). The significance of the auxiliary function of MMAA is evident in that mutations in this protein lead to the birth defect, type A MMA (13, 34). Recent studies indicate that MeaB function is to promote the activity of MCM (7, 9, 10), perhaps by assisting in the loading of coenzyme B12 into MCM, and/or protecting the B12 cofactor from oxidative damage during MCM turnover (7, 9).

The solution of the crystal structure of GDP-MeaB confirms that it is a member of the G3E (metallochaperone) family of GTPases. Motifs responsible for nucleotide binding and signal communication have been identified. While the existence of an intact NKXD guanine specificity motif and the presence of a glutamate rather than aspartate in switch II (Walker B) motif firmly establish MeaB as a G3E GT-Pase, MeaB differs from other metallochaperones HypB, UreG, and CooC, in containing unique N- and C-terminal extensions from the G domain. Here, the crystal structure reveals the importance of the C terminus in forming nucleotide-independent homodimers, correlating well with the observation that another G3E GTPase, HypB, which lacks this extension, dimerizes in a nucleotide-dependent fashion (5). One of the surprises of this structure determination is how this C-terminal extension creates such a different dimer interface for MeaB compared with HypB. Given that this C-terminal extension is not found in the other metalloenzyme chaperones, we predict that other G3E subfamily members will dimerize in a nucleotide-dependent fashion like HypB. The N-terminal extension, which is also unique to MeaB is more difficult to rationalize; given its location on the surface of the protein, the most appealing postulate is that this region is required for interaction with MCM, a role that other G3E proteins do not have.

One of the more exciting uses of the MeaB structure is as a framework for understanding the structural basis of the pathology of type A MMA. Some of the mutations that lead to type A MMA involve atypical structural features. For example, R145Q and R357Q mutations affect a rather unusual 4-arginine patch that spans the G domain and C-terminal region. Attempts to express the corresponding R57Q and R272Q mutant forms of MeaB result in accumulation in inclusion bodies, consistent with these proteins being in unfolded aggregate states. Therefore, this 4-arginine patch and its corresponding negatively charged amino acid counterparts must play a role in MeaB stability. The F119C mutation is also intriguing, since the structure suggests, and mutagenesis confirms, that this MMA-associated mutation can lead to the creation of a disulfide bond. Mutations that disrupt disulfide bonds are known to be harmful, but a mutation that creates a disulfide bond is certainly more rare. The last three type A MMA mutations are more common; a mutation of a residue to proline (L11P) that the MeaB crystal structure shows will disrupt a helix, and two mutations of glycines to glutamates (G147E and G218E). Mutations of glycine residue are often disruptive to a protein structure, as are mutations of neutral residues to negatively charged ones. In one case, G147E, the corresponding glycine in MeaB (Gly29) is buried with somewhat unusual Ramachandran angles, making it easy to understand why this mutation is deleterious. In the other, G218E, the corresponding glycine in MeaB (Gly130) is partially solvent exposed and in an α-helix, making it difficult to explain why these mutations result in the observed protein instabilities. It is interesting that so many of the MMA-related mutations affect protein stability. This trend may reflect the importance of the protein-protein interaction between MeaB and MCM, and the robustness of that interaction to most single site mutations. For enzymes with complex function, a single mutation in an active site can abolish activity. For chaperones, more than one change in a surface residue is expected to be required to preclude binding to its target enzyme. Thus, “killing” the function of a chaperone with a single mutation may require that that mutation has a deleterious affect on the protein structure or its stability. As more data becomes available about genetic disorders in chaperone proteins, it will be interesting to see if these observations represent a general trend.

Biochemical data support the idea that the GTPase activity of MeaB assists in the loading of coenzyme B_{12} into MCM (10). The structure of GDP-MeaB presented here must not represent the active GTPase form of MeaB, since the regions that should communicate GTP hydrolysis (switch I and II) are not accessible to MCM in this structure. Several pieces of evidence suggest the conformation of MeaB changes between GDP- and GTP-bound states; dynamic light scattering experiments suggest that MeaB undergoes a significant conformational change upon nucleotide binding (9), and the apparent surface area of MeaB buried by MCM differs depending on the nucleotide-bound state of MeaB. Also, our inability to introduce a GTP analogue into MeaB, either by cocrystallization under native crystallization conditions or soaking methods, is consistent with the idea that a conformational change in MeaB is required for GTP substrate binding. We predict that in the GTP-bound form of MeaB, the switch I and II regions are exposed to MCM, as is the MeaB N-terminal extension, and that the signal produced by
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GTP hydrolysis is likely to assist in obtaining a form of MCM to which coenzyme B12 can readily bind. The second proposed function of MeaB, to protect B12 from oxidative inactivation is facilitated in the presence of GTP, and to a lesser extent GDP, but does not require GTP hydrolysis. If this proposed activity is simply due to a substantial binding interaction between MeaB and MCM to sequester the cofactor from solvent, it makes sense that the GDP-bound form only contributes a 3-fold protection compared with a 15-fold effect for the GTP-bound form, as no extended surface exists in the GDP-MeaB structure that could form substantial interactions with MCM. Consistent with the results of biochemical binding studies, GDP-MeaB buries only ~4000 Å2 of surface of the holo-MCM compared with the 7000 Å2 buried in GTP-MeaB. Again, we would predict that the protection of GDP-MeaB has more extended surface area available for interaction with MCM.

For many decades, enzymologists have focused on the fascinating chemistry performed by metalloenzymes. The amazing 1,2 rearrangements catalyzed by some coenzyme B12 enzymes is just one example. Now scientists are finding that the auxiliary proteins that play roles in metallo cofactor protection and assembly are just as interesting as the metalloenzymes themselves. With MeaB representing only the third structure of a G3E metallochaperone GTPase family member (5, 36), we are just starting to explore the structure/function relationships for what promises to be a fascinating protein family.

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