Structure and Mechanism of the Rebeccamycin Sugar 4′-O-Methyltransferase RebM*

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The 2.65-Å crystal structure of the rebeccamycin 4′-O-methyltransferase RebM in complex with S-adenosyl-l-homocysteine revealed RebM to adopt a typical S-adenosylmethionine-binding fold of small molecule O-methyltransferases (O-MTases) and display a weak dimerization domain unique to MTases. Using this structure as a basis, the RebM substrate binding model implicated a predominance of nonspecific hydrophobic interactions consistent with the reported ability of RebM to methylate a wide range of indolocarbazole surrogates. This model also illuminated the three putative RebM catalytic residues (His\(^{140}\) and Asp\(^{166}\)) subsequently found to be highly conserved among sequence-related natural product O-MTases from GC-rich bacteria. Interrogation of these residues via site-directed mutagenesis in RebM demonstrated His\(^{140}\) and Asp\(^{166}\) to be most important for catalysis. This study reveals RebM to be a member of the general acid/base-dependent O-MTases and, as the first crystal structure for a sugar O-MTase, may also present a template toward the future engineering of natural product MTases for combinatorial applications.

The indolocarbazole alkaloids are typically divided into two major classes dependent upon their structure and mechanism of action (1–3). Specifically, the staurosporine-type indolocarbazoles (Fig. 1A, 1 and 2) possess indole nitrogens bridged by a single glycosyl moiety at C-1′ and C-5′ and are potent inhibitors of protein kinases A, C, and K (4), whereas the rebeccamycin-type variants (Fig. 1A, 3 and 4) are distinguished by a β-glucoside attachment critical for the potent topoisomerase I poisoning effects and notable anticancer activities of this class (5–7). Biosynthetically the indolocarbazoles are derived from tryptophan, glucose, methionine, and, in the case of the rebeccamycin-type indolocarbazoles, chloride (5, 8). The biosynthetic gene clusters encoding 1, 3, and 4 have been reported (9–11), and a variety of in vitro and in vivo studies have contributed to an understanding of the enzymes responsible for indolocarbazole core biosynthesis (Fig. 1B). As exemplified by the rebeccamycin core reactions these include RebI-catalyzed tryptophan halogenation (12), RebO/RebD-mediated chomopyrrole acid formation (12–14), and RebC/RebP-catalyzed ring closure (5, 15, 16). Similar studies have delineated the catalysts responsible for the key structural features that mechanistically distinguish the indolocarbazoles including the enzymes to form and attach novel sugars (11, 17, 18) and a culminating series of S-adenosylmethionine (AdoMet)\(^{3}\)-dependent N- and O-methylations (11, 17, 18). Among this set of final tailoring reactions, the differential sugar O-alkylation provided by RebM, AtM, and StaMB greatly influences the biological activity of the corresponding indolocarbazoles (17).

The first indolocarbazole sugar O-methyltransferase (O-MTase) to be characterized in vitro, RebM catalyzes glucosyl C-4′-O-methylation as the final step in rebeccamycin biosynthesis (Fig. 1B) (17). RebM displays some subtle distinctions from the other sugar O-MTases studied in vitro including oleandomycin OleY (19), tylosin TyI and TyIF (20, 21), mycaminic MycF (22), coumermycin CouP (23), and novobiocin NovP (24). In contrast to typical multimeric methyltransferases (19–21, 25–27), RebM functions as a monomer. Unlike OleY, TyI, and TyIF, RebM activity is observed over a broader pH range and cannot be enhanced by divalent metals. However, the most striking unique feature of RebM is its ability to accept a wide range of alternate substrates. Specifically, in addition to the native substrate (Fig. 1B, 13), RebM can tolerate variation on the imide heterocycle, deoxygenation of the sugar moiety, and even indolocarbazole glycoside anomers (17). Furthermore, RebM was the first secondary metabolite-associated MTase demonstrated to utilize non-natural cofactor analogs of AdoMet, such as synthetic N-mustard adenylates, to provide novel adenylated indolocarbazole conjugates (28). Similar to DNA MTases in recent reports (29–31), RebM can also accomplish differential alkylation (replacing methyl with alkyl) in the presence of the appropriate AdoMet analogs. Thus, RebM, and natural product MTases in general, may present spectacu
lar opportunities for rapid enzymatic diversification of therapeutically important complex natural products.

In an effort to further our understanding of the mechanism of RebM catalysis and the key structural elements for AdoMet recognition and activation, we report the crystal structure of RebM at 2.65 Å. This work revealed RebM to crystallize as a dimer and adopt a fairly typical MTase structural fold. The location and nature of the putative RebM dimer interface is unique in comparison with other MTases and, based upon gel filtration studies, is likely not critical for catalysis. Site-directed mutagenesis of key residues in the AdoMet-binding pocket revealed some tolerance to variation with little overall alteration in structure, AdoMet binding, and/or catalysis. Mutagenesis studies also identified key catalytic residues and confirmed RebM to be a member of the acid/base-dependent O-MTases.

As the first crystal structure for a sugar O-MTase, this work may also present a template toward the future engineering of natural product MTases for combinatorial applications.

**MATERIALS AND METHODS**

**Protein Expression and Purification**—Lechevalieria aerocolonigenes RebM and all engineered RebM mutants were produced as amino-terminal His<sub>6</sub> fusion proteins using the expression plasmid pCST28aRebM in Escherichia coli BL21(DE3)pLysS (17). The culture was grown (28 °C at 250 rpm) to midlog phase (A<sub>600</sub> = 0.6) at which point isopropyl 1-thio-β-D-galactopyranoside was added to a 0.4 mM final concentration. Growth was continued for an additional 4–6 h, the cells were collected by centrifugation (15 min at 5000 × g), and resuspended in 50 mM sodium phosphate, pH 8.0, containing...
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300 mM NaCl and 20 mM imidazole on ice. The cells were lysed via incubation with 1 mg/ml lysozyme (~50,000 units/mg Sigma) for 30 min on ice followed by sonication (VirSonic 475; Virtis, Gardiner, NY; 100 watts, 4 × 30-s pulses, ~1 min between pulses) on ice. Protein was purified with nickel-nitrilotriacetic acid-agarose resin or spin columns (Qiagen, Valencia, CA) using the manufacturer’s protocols. The buffer was exchanged with 20 mM potassium phosphate, pH 8, via PD-10 gel filtration columns (GE Healthcare), and the purified enzyme was subsequently concentrated to >10 mg/ml, drop frozen in liquid nitrogen, and stored at ~80 °C. Protein concentrations were determined by Bradford assay (Bio-Rad) using bovine serum albumin as a standard. For the production of selenomethionine-labeled protein, the E. coli methionine auxotroph strain B834 (DE3) was transformed with the plasmid, and autoclithionyl-labeled protein, the serum albumin as a standard. For the production of selenomethionine-labeled protein, the E. coli methionine auxotroph strain B834 (DE3) was transformed with the plasmid, and auto-

TABLE 1
Data collection and refinement statistics for RebM-AdoHcy complex

| Space group    | P4₃2₁2       |
|----------------|--------------|
| Unit cell parameters (Å) | a = b = 119.2, c = 84.4 |
| **Collection statistics** | | |
| Wavelength (Å) | 0.97869 |
| Resolution range (Å) | 84.22–2.65 (2.74–2.65) |
| No. of reflections (measured/unique) | 458,839/17,746 |
| Completeness (%) | 97.2 (81.3) |
| Rmerge | 0.074 (0.382) |
| Reduction | 25.9 (14.5) |
| Mean I/σ | 41.7 (3.4) |
| **Phasing statistics** | | |
| Phasing power | 1.823 |
| Mean FOM (centric/acentric) | 0.131/0.418 |
| Rmerge | 0.629 |
| **Refinement statistics** | | |
| Resolution range (Å) | 84.22–2.65 |
| No. of reflections (total/test) | 16,813/901 |
| Rcryst | 0.214 |
| Rfree | 0.260 |
| r.m.s.d. bonds (Å) | 0.011 |
| r.m.s.d. angles (°) | 1.330 |
| ESU from Rfree | 0.335 |
| No. of protein molecules/all atoms | 2/3,709 |
| No. of waters | 6 |
| *Ramachandran plot by PROCHECK (%) | | |
| Core region | 90.6 |
| Allowed region | 9.2 |
| Generously allowed | 0.2 |

* Rmerge = ΣΣ|I(h) − I(h)/ΣΣI(h) where I(h) is the intensity of an individual measurement of the reflection and I(h) is the mean intensity of the reflection.
* Rcryst = ΣΣ|Fobs − |Fcalc|ΣΣFcalc| where Fobs and Fcalc are the observed and calculated structure factor amplitudes, respectively.
* Rfree was calculated as Rcryst using 5.0% of the randomly selected unique reflections that were omitted from structure refinement.

Protein Crystallization—RebM was crystallized by the hanging drop vapor diffusion method at 293 K. The reservoir solution contained 8% (w/v) methyl ether polyethylene glycol 5000, 200 mM ammonium sulfate, and 100 mM sodium acetate, pH 5.0. The hanging drop consisted of 2 μl of protein solution (12 mg/ml RebM, 50 mM sodium chloride, and 10 mM Tris-HCl, pH 8.0) mixed with 2 μl of reservoir solution. RebM crystals (rectangular, ~350 × 50 × 30 μm in size) required 1 month to achieve full size. The crystals were subsequently soaked in increasing concentrations of ethylene glycol in mother liquor up to a final concentration of 20% (v/v) and flash frozen in a stream of liquid nitrogen.

Data Collection and Reduction—X-ray diffraction data were collected at the Advanced Photon Source on Life Sciences Collaborative Access Team beamline 21-ID-D at a temperature of 93 K. Reflections were indexed, integrated, and scaled using the HKL2000 package (33).

Structure Determination—A total of 12 of 14 potential selenium sites were identified with HySS (34, 35). The data were phased via single wavelength anomalous dispersion using autoSHARP with the help of auxiliary programs from the CCP4 suite (36, 37). Coot (38) was used to manually build into the density-modified map produced by autoSHARP. Once a sufficient number of residues were assembled within both RebM molecules of the asymmetric unit to accurately determine the noncrystallographic symmetry operator, density modification was carried out with RESOLVE resulting in a higher quality electron density map (39). The structure was then completed through multiple rounds of model building with Coot and refinement with REFMAC (40). TLS (translation/libration/screw) groups were incorporated during the final stages of refinement. These groups were selected based on the output of the TLSMD web server (41). Relevant crystallographic statistics are summarized in Table 1.

Energy Minimization—The substrate complex model was generated by manual insertion of 13 into the suspected active site. The simulation assumed an S₄,2 mechanism and was based upon the closely related mycolic acid synthase-mycolic acid ternary complex (42) (Protein Data Bank code 1KPI). In the superimposed model, 13 was positioned in the cavity above the AdoMet binding site in an equivalent position of mycolic acid, and the 13 4’-hydroxyl was linearly aligned with respect to the methyl donor to facilitate the nucleophilic attack. Model refinement relied upon 200 steps of conjugate gradient minimization as implemented in the CNS (Crystallography and NMR System) model minimization procedure (43). Topology and parameter files for 13 and S-adenosylhomocysteine (AdoHcy) were obtained from the HIC-Up server (44).

Site-directed Mutagenesis—RebM mutants were generated with the QuikChange II mutagenesis kit (Stratagene, La Jolla, CA) using the parent expression plasmid pCST28aRebM as template (17). The mutagenic primers are listed in supplemental Table S1. All mutant plasmids were confirmed by DNA sequencing to carry the desired mutations. Synthesis of oligonucleotide primers and sequencing of DNA were performed at the University of Wisconsin-Madison Biotech Center. Plasmids containing the confirmed rebM mutations were then transformed into E. coli BL21(DE3), and the corresponding overproduced recombinant mutant proteins were purified as described for the wild-type enzyme.

CD Spectroscopy—To confirm the influence of the targeted RebM mutations upon the global protein fold, all mutant proteins were analyzed by CD spectroscopy. For CD analysis, the mutant proteins were exchanged at 4 °C with buffer (50 mM phosphate, pH 8), and the protein concentration of each sample was subsequently determined by the Bradford assay (Bio-Rad). An AVIV model 202 circular dichroism spectrometer (AVIV Associates, Lakewood, NJ) was used to record the CD spectra using a 1-mm-path length quartz cell containing 300 μl of each protein sample (~0.25 mg/ml) maintained at 25 °C in a ther-
Enzymatic Reactions—The enzyme assay was accomplished as reported previously (17). Reactions were performed in phosphate buffer (50 mM, pH 8.0) containing 50 μM 13 (Fig. 1B), 20 μM purified RebM, and 100 μM AdoMet (Sigma) with incubation at 30 °C for 4 h in a total volume of 20 μl. All reactions were quenched by the addition of 20 μl of methanol followed by centrifugation (10 min at 10,000 × g) to remove precipitated protein. Reactions were analyzed using an analytical Varian ProStar high pressure liquid chromatography system (Phenomenex Luna C_{18}, 5 μm, 250 × 4.6 mm, 0.1% aqueous trifluoroacetic acid with a gradient of 10–100% CH_{3}CN over 20 min at 1.0 ml/min, A_{216}), and reaction products were confirmed as described previously (17).

Kinetic Measurements—Pseudo first-order kinetics were assessed under constant AdoMet concentration (320 μM) and variable 13 concentrations (2.5, 5, 10, 25, 50, and 100 μM) and under constant 13 concentration (50 μM) and variable AdoMet concentrations (0.008, 0.032, 0.16, 0.32, 1.6, and 3.2 mM). Assays were conducted in triplicate, and all rates were confirmed to be linear. The kinetic curves were fit to the Michaelis-Menten equation using SigmaPlot with the Enzyme Kinetics module (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

Quality of the Maps and the Model—RebM, a 30-kDa protein consisting of 273 amino acids, crystallized as a homodimer, and the structure was refined to a nominal resolution of 2.65 Å. The asymmetric unit contained two protein molecules (labeled A and B), one molecule of S-adenosylhomocysteine per protein, and six ordered water molecules. The first 42 residues of chain A and the first 46 residues of chain B (20 residues of which comprised the amino-terminal His tag in each case) were not modeled because of insufficient electron density. In addition, the surface loop of chain B containing residues 35–40 could not be modeled. The α carbons of the two chains align with a root mean square deviation of 0.514 Å. The final structure was refined to an R_{cryst} and R_{free} of 21.4 and 26.0%, respectively. The slightly high residual values as well as the low ordered solvent content may be related to the extremely strong diffuse scattering observed in the diffraction images. This diffuse scattering was also observed during room temperature data collection suggesting that it was inherent to the crystals and not the result of cryoprotection. The RebM crystals described belong to the space group P4_{3}2_{1}2 with unit cell parameters a = b = 119.2 Å, c = 84.4 Å.

Overview of the Structure—In the RebM crystal structure, the two monomers in the homodimer are related by 2-fold crystallographic symmetry (Fig. 2A). The dimer interface in previously reported small molecule MTases is typically found above the substrate binding site and often contains an extra amino-terminal dimerization domain also important for catalysis. In contrast, the RebM dimer interface is composed predominantly of hydrogen bonding interactions encompassing residues Thr^{211}–Asp^{215} of β-strand-6 aligned in an antiparallel orientation (Fig. 2A). Four backbone-backbone amide-carbonyl hydrogen
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| POS | Protein Data Bank code | Zb | r.m.s.d. | LALId | %IDE | Description |
|-----|------------------------|----|---------|--------|------|-------------|
| 1   | 2OS7                   | 28.2| 1.8     | 237    | 295  | Putative sarcosine dimethylglycine N-methyltransferase, SDMT (G. sulfurarum) |
| 2   | 2FK7                   | 27.2| 2.0     | 241    | 281  | Methoxy mycolic acid synthase, Hma (M. tuberculosis) |
| 3   | 1KP9                   | 24.9| 2.2     | 233    | 270  | Mycolic acid cyclopane synthase, CmaA1 (M. tuberculosis) |
| 4   | 1T2W                   | 17.6| 2.7     | 180    | 340  | Carminomycin 4-O-methyltransferase, DnrK (S. peucetius) |
| 5   | 1QZZ                   | 17.2| 2.8     | 180    | 340  | Aclacinomycin 10-hydroxylation, RdmB (Streptomyces pseudopurpurascens) |
| 6   | 1Y8C                   | 16.9| 2.5     | 168    | 244  | S-Adenosylmethionine-dependent methyltransferase (Clostridium acetobutylicum ATCC 824) |
| 7   | 1RIS                   | 16.9| 3.1     | 192    | 252  | 5-mRNA cap (guanine N-7) methyltransferase (Eucephalotozoon cuniculi) |
| 8   | 1IM8                   | 16.9| 3.1     | 180    | 225  | YecO methyltransferase (Haemophilus influenzae, Hid019) |
| 9   | 1XVA                   | 15.0| 3.0     | 170    | 239  | Glycine N-methyltransferase, GNMT (Rattus norvegicus) |
| 10  | 1HNN                   | 15.0| 2.8     | 183    | 261  | Phenylethanolamine N-methyltransferase, PNM1 (Homo sapiens) |
| 11  | 1FPX                   | 14.5| 4.8     | 131    | 175  | Isoflavone O-methyltransferase, IOMT (Medicago sativa) |
| 12  | 1FX9                   | 14.5| 2.9     | 183    | 231  | Thiopurine S-methyltransferase, TPMT (Mus musculus) |
| 13  | 1TPQ                   | 14.3| 3.7     | 166    | 333  | Chalcone O-methyltransferase, ChiOMT (M. sativa) |
| 14  | 1M6E                   | 13.6| 3.6     | 203    | 359  | Salicylic acid carboxyl methyltransferase, SAMT (Clarkia breweri) |
| 15  | 1T43                   | 13.4| 2.5     | 157    | 274  | Protein methyltransferase, HemK (E. coli) |
| 16  | 1KXZ                   | 13.4| 4.0     | 159    | 178  | Precorrin-6y methyltransferase, MT0146/CbiT (Methanothermobacter thermoautotrophicus) |
| 17  | 1SU1                   | 13.2| 3.0     | 171    | 227  | Caffeoyl-CoA-O-methyltransferase, CCoAMT (alfalfa) |
| 18  | 1VID                   | 12.5| 2.9     | 162    | 214  | Catechol O-methyltransferase, COMT (R. norvegicus) |

a Position in the numerical listing of structural homologs.

b Z-score; strength of structural similarity in standard deviations above expected.

Positional root mean square deviation of superimposed Cα atoms in Å.

Total number of equivalenced residues.

Length of the entire chain of the equivalent structure.

Percentage of sequence identity over equivalent positions.

bonds are involved in the dimerization interface (Fig. 2A), the span of which is quite small overall, comprising only 380 Å² of the buried surface area (~3% of the total surface area). Consistent with gel filtration studies that revealed RebM to be a monomer in solution (17), the relatively small size of the unique RebM dimer interface suggests it to be of low affinity. This is unlike the previously reported small molecule MTases (such as carminomycin 4-O-methyltransferase (DnrK) (27), 5α-O-carboxymethylecyclophosphorin C methyltransferase (Cmcl) (45), chalcone O-methyltransferase (26), isoflavone O-methyltransferase (26), and caffeic acid/5-hydroxyferulic acid 3/5-O-methyltransferase (46)) wherein the dimer interface is comprised of nearly 30% of the total surface area. Because the dyad-related monomer of RebM does not contribute to the active site of its partner molecule, dimerization does not appear to be necessary for the substrate recognition or transmethylation. In addition to RebM, only two other MTases invoke an atypical β-strand-6 dimer interface. Of these, the caffeoyl-coenzyme A 3-O-methyltransferase (47) β-strands of the dimer interface adopt a parallel orientation, whereas the analogous β-strands in sarcosine dimethylglycine methyltransferase (Protein Data Bank code 2057) align in a RebM-like antiparallel fashion but, unlike RebM, form four salt bridges. In the context of catalysis, caffeoyl-coenzyme A 3-O-methyltransferase dimerization is not critical for activity, whereas the catalytic influence of sarcosine dimethylglycine methyltransferase dimerization has not been reported.

Each subunit of RebM folds into a single globular domain responsible for binding AdoMet and the substrate. The domain exhibits a common tertiary structure consisting of a core α/β Rossmann fold, which is characteristic of all other AdoMet-dependent MTases (48), and a unique α-helical cap that forms the top of the active site cavity. In the overall subunit structure, a central parallel β-sheet (β1–β7) is located between helices (α1–α3, α5, and α7) (Fig. 2B). The β-sheet has strand topology β3, β2, β1, β4, β5, β7, β6 containing a single antiparallel strand (β7) and packs against three proximal (α1–α3) and two distal (α5 and α7) helices. Five helices (α4, α6, and α8–α10) form the upper cap of the globular domain, and helices analogous to the latter three commonly interact with the substrates in MTase homologs.

Structural Homology—Although this work represents the first reported sugar O-MTase crystal structure, based upon the Research Collaboratory for Structural Bioinformatics (RCSB), over 260 functionally diverse MTases have been characterized structurally (49, 50). These enzymes show no or very low overall sequence identity to each other, but most share a common AdoMet-dependent MTase fold. Many members also contain additional domains outside the core MTase structure that play a role in substrate recognition or alternative functions.

A DALI search for structures similar to that of RebM returned several hits with Z-scores of >12 (Table 2), including cyclopropane synthase (Cmaa1) (42), methoxy mycolic acid synthase (Hma) (51), sarcosine dimethylglycine and glycine methyltransferase (Protein Data Bank codes 2057 and 1XVA) (52), DnrK (27), aclacinomycin hydroxylase (RdmB) (53), isoflavone O-methyltransferase (26), caffeic acid/5-hydroxyferulic acid 3/5-O-methyltransferase (54), chalcone O-methyltransferase (26), caffeoyl-coenzyme A 3-O-methyltransferase (47), and phenylethanolamine N-methyltransferase (55). This entire panel shares very low overall sequence identity (~7–23%) but high sequence conservation among residues associated with AdoMet binding, specifically the core fold between β1 and β2 that interacts with the AdoMet homocysteine and ribosyl moieties and an acidic residue in the loop between β2 and β3 that interacts with the exocyclic N⁶ and ring N-1 of the adenine ring of the cofactor. Although most of the RebM struct-

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homologs, sarcosine dimethylglycine methyltransferase (Protein Data Bank code 2K8F) and the mycolic acid synthase Hma (51), revealed an overlap with root mean square deviation of 1.8 and 2.0 Å for 237 and 241 Cα atoms, respectively (Fig. 3, A and B).

**AdoMet/AdoHcy Binding Site**—The electron density for the cofactor AdoHcy (demethyl-AdoMet) is well defined in the ReBM structure. The AdoMet/AdoHcy binding site is located in the carboxyl-terminal end of a cleft formed by the β-strands highly conserved throughout MTases. AdoHcy is bound through an extensive hydrogen bond network and van der Waals interactions and is partially exposed to solvent (Fig. 2B).

Specifically the ReBM-AdoHcy interaction mainly engages residues in the loops between β1 and β2 (L1; binds the amino acid and ribosyl moieties), between β2 and β3 (L2; contacts ribose and adenine), and after β3 (L3; interacts with adenine) and also helix α4 (binds the amino acid and adenine). The adenine ring of AdoHcy is situated in a hydrophobic pocket formed by the aliphatic side chains Ile92 (L2), Ala120 (L3), and Met142 (helix α1). The ribosyl moiety is anchored via a hydrogen bond from the C-2′ and C-3′ hydroxyl groups to the side chain of Glu129 (L2), Ala130 (L3), and Met142 (helix α4). The exocyclic N6 and ring N-1 of the adenine ring are hydrogen-bonded to the side chain carboxyl oxygen of Asp119 (L3). The ribosyl moiety is anchored via hydrogen bonds from the C-2′ and C-3′ hydroxyl groups to the side chain of Glu129 (L2), Ala130 (L3), and Met142 (helix α4). The exocyclic N6 and ring N-1 of the adenine ring are hydrogen-bonded to the side chain carboxyl oxygen of Asp119 (L3).

**FIGURE 3.** Comparison of the overall topologies of homologous. A, structural overlay of ReBM from *L. aerocolonigenes* (Protein Data Bank code 3BUS) colored pink and sarcosine dimethylglycine methyltransferase from *Galdieria sulfuraria* (protein Data Bank code 2SOY) colored green (root mean square (RMS) deviation between the structures is 1.8 Å), B, structural overlay of ReBM from *L. aerocolonigenes* (Protein Data Bank code 3BUS) colored pink and mycolic acid synthase Hma (Mmaa4) from *Mycolibacterium tuberculosis* (Protein Data Bank code 2K8F) colored blue (root mean square (RMS) deviation between the structures is 2.0 Å), AdoHcy (SAH) is in stick model and colored yellow. The molecular graphics program PyMOL was used in generating these graphics. C, sequence alignment of ReBM from *L. aerocolonigenes* (Protein Data Bank code 3BUS), sarcosine dimethylglycine methyltransferase (GNMT, glycine N-methyltransferase) from *Galdieria sulfuraria* (Protein Data Bank code 2SOY), mycolic acid synthase Hma (Mmaa4) (Protein Data Bank code 2K8F) from *M. tuberculosis*, mycolic acid cyclopropane synthase (Cmaa1) (Protein Data Bank code 1K9M) from *M. tuberculosis*, and caminomycin 4-0-methyltransferase (DmrK) (Protein Data Bank code 1TWW) from *Streptomyces peucetius* in this alignment, the predicted secondary structure is illustrated above the sequence. Helices and sheets are colored red and green, respectively, whereas yellow-colored helices α8, α9, and α10 indicate incomplete sequence. Loops involved in cofactor binding are labeled (L1–L3), and sequence of blue color designates the AdoMet binding core domain (α7–α10). Highly or moderately conserved residues are colored red and orange, respectively, with putative catalytic residues (based upon ReBM mutagenesis) colored green.

### TABLE 3

| Mutant | *k*<sub>cat</sub>/| AdoMet | 13 *k*<sub>cat</sub> | Mutant | *k*<sub>cat</sub>/| wt |
|--------|-----------------|--------|-----------------|--------|-----------------|----|
|        | *K*<sub>m</sub> | *K*<sub>m</sub>| *K*<sub>m</sub>|        | *K*<sub>m</sub>| *K*<sub>m</sub>| *K*<sub>m</sub>|        | *K*<sub>m</sub>| *K*<sub>m</sub>| *K*<sub>m</sub>|        | *K*<sub>m</sub>| *K*<sub>m</sub>| *K*<sub>m</sub>|        | *K*<sub>m</sub>| *K*<sub>m</sub>| *K*<sub>m</sub>|        | *K*<sub>m</sub>| *K*<sub>m</sub>| *K*<sub>m</sub>|        |
|        | μM | μM | μM |        | μM | μM | μM |        | μM | μM | μM |        | μM | μM | μM |        | μM | μM | μM |        | μM | μM | μM |        | μM | μM | μM |        |
| Wild type | 2.8 ± 0.3 | 12 ± 2 | 35.5 ± 7.4 | 1 | C70A | 2.9 ± 0.3 | 17 ± 3 | 22.5 ± 4.6 | 1.03 | C70S | 4.3 ± 0.4 | 34.5 ± 2.7 | 17 ± 4 | 1.53 | P75S | 0.38 ± 0.05 | 162 ± 35 | 53.5 ± 14.2 | 0.13 | W134Y | 1.00 ± 0.08 | 32 ± 3 | 16 ± 3 | 0.35 | L136V | 1.52 ± 0.03 | 56 ± 5 | 21.5 ± 3.8 | 0.54 | S138A | 1.39 ± 0.04 | 18 ± 3 | 21 ± 4 | 0.49 | H140A | 0.14 ± 0.01 | 31.1 ± 26 | 112.2 ± 1.9 | 0.05 | H141A | 0.60 ± 0.03 | 94.3 ± 8.6 | 79.8 ± 11.5 | 0.21 | D166A | 0.30 ± 0.02 | 162 ± 30 | 31.99 ± 2.5 | 0.10 | H140A/H141A | ND | ND | ND |

*ND, activity not detected (below the detection limit; <0.01 nmol min⁻¹ mg⁻¹ of the enzyme).*
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Substrate Binding Model—Attempts to obtain a complex with bound rebeccamycin (15) or 13 (in the presence or absence of AdoMet) by co-crystallization were ineffective. The binding of 13 within the ternary RebM-AdoHcy-13 complex was therefore simulated by docking. In this model, 13 was aligned with the activated AdoMet methyl in an orientation consistent with a postulated S_{N}2 attack by the nucleophilic sugar 4'-hydroxyl group (Fig. 2D). Based upon this model, the proposed binding site for 13 is encapsulated by α-helices α6, α8, α9, and α10, and substrate binding is dominated by hydrophobic and van der Waals interactions involving residues Phe^{254}, Leu^{224}, Ala^{183}, Phe^{184}, Ala^{190}, Val^{191}, Phe^{207}, Tyr^{260}, Phe^{30}, Leu^{28}, and Trp^{39}. Only a few putative hydrogen bond participate in anchoring the substrate, potentially derived from residues Glu^{137} and Asp^{166} (Fig. 2D). Specifically the sugar O-2' and O-3' are within hydrogen bonding distance with the Asp^{166} side chain carboxylate, whereas O-2' is also poised to interact with the Glu^{137} side chain carboxylate. Although only a simulation, the predominance of non-specific hydrophobic interactions with only a few specific hydrogen bonds is arguably consistent with the previously reported ability of RebM to methylate a wide array of structurally diverse substrates (17).

Reaction Mechanism—Typically MTases catalyze S_{N}2-like reactions involving oxygen-, nitrogen-, and carbon-based nucleophiles that require at least one proton transfer step prior to, in concert with, or after methyl group transfer. In most methyltransferases, acid/base catalysis contributes to rate acceleration wherein an active site His participates as a catalytic base as exemplified by chalcone O-MTase, isoflavone O-MTase, and arginine N-methyltransferase (PRMT3) (26, 56). Alternatively, in carminomycin 4-O-MTase (DnrK) mutagenesis of the one residue (Tyr^{142}) in close proximity (3 Å) of the substrate hydroxyl revealed this residue to only moderately influence catalysis. Thus, it was concluded that, unlike most MTases, DnrK and its mechanistically related counterparts, such as mRNA cap guanine N-7 N-MTase and Gly N-MTase, are entropic enzymes that rely upon proximity and orientation of cofactor mutants displayed enzymatic activity similar to that of the wild-type RebM. Mutant P7SS (Table 3), although properly folded based upon CD spectroscopy (see supplemental Fig. 1), displayed a substantially reduced affinity for AdoMet (>10-fold increase in $K_{D}$) and a parallel 11-fold decrease in catalytic efficiency ($k_{cat}/K_{m}$), highlighting the importance of the Pro^{75} side chain in anchoring the carboxyl terminus of AdoHcy.
effects for rate enhancement (27). A third MTase mechanism, as exemplified by caffeoyl-CoA O-methyltransferase (47), invokes a divalent metal-dependent process. All attempts to align RebM with MTases for which the catalytic residues have been mapped were unsuccessful. However, the RebM substrate binding model (Fig. 2D) notably implicated two histidines (His\textsuperscript{140/141}; helix α4) as structural equivalents to the catalytic histidines of isoflavone O-methyltransferase and chalcone O-methyltransferase. In the RebM model, both His\textsuperscript{140/141} are within range (2.8–3.4 Å) to deprotonate the 4′-hydroxyl of 13 (pK\textsubscript{a} ~ 16). Of these two putative catalytic residues, His\textsuperscript{140} is also within hydrogen bonding distance (3.01 Å) of Asp\textsuperscript{166}. Consistent with a putative acid/base mechanism for RebM, EDTA or divalent metals do not influence RebM activity (17).

Interrogation of the three putative catalytic residues via mutagenesis revealed decreases in the apparent $K_{cat}$ by 20–10– and 4-fold for H140A, D166A, and H141A, respectively (Table 3). Both D166A and H141A also displayed significant increases in the apparent $K_{m}$ for AdoMet (≥8-fold), whereas H141A also led to a ~2-fold reduction in affinity, based upon the apparent $K_{m}$ for 13. In contrast, a slight decrease in the apparent $K_{m}$ for AdoMet (~3-fold) and a moderate increase in the apparent $K_{m}$ for 13 (~2.5-fold) were observed for mutant H140A. Evaluation of the H140A/H141A double mutant revealed a properly folded (based upon CD), but inactive, protein. These results are consistent with His\textsuperscript{140} as the preferred general base potentially constrained by interactions with AdoMet (166), a residue that, although positioned ~7–10 Å from AdoMet in the static structural model, is critical for AdoMet binding based upon kinetic characterization. Interestingly, in isoflavone O-methyltransferase, the corresponding general base (His\textsuperscript{277}) is constrained and oriented via hydrogen bonding with a specific glutamate (Glu\textsuperscript{316}). Given this precedent and the significant reduction of the apparent $K_{cat}$ in RebM D166A, we postulate that RebM Asp\textsuperscript{166} orients the RebM general base His\textsuperscript{140} in a similar manner. Consistent with the position of His\textsuperscript{141} at the AdoMet-13 interface in the static structural model, mutagenesis of His\textsuperscript{141} reduced the apparent $K_{m}$ for both AdoMet and 13. In the context of RebM H140A, His\textsuperscript{141} may also weakly compensate as a general base, as the double mutant H140A/H141A is completely inactive. Notably alignment of RebM with natural product O-MTases from GC-rich bacteria (Fig. 4) revealed His\textsuperscript{140/141} and Asp\textsuperscript{166} to be invariant residues among this set of enzymes. Based upon this analysis, we propose these invariant residues to play similar catalytic roles (His\textsuperscript{140/141} equivalents as the general base potentially constrained by interactions with Asp\textsuperscript{166} homologs) in each of these respective MTases.

**Summary**—The determination of the crystal structure of RebM in complex with S-adenosyl-L-homocysteine enabled the illumination of key catalytic residues (His\textsuperscript{140/141} and Asp\textsuperscript{166}) that, based upon this study, were found to be invariant among a wide array of natural product O-MTases from GC-rich bacteria. This study revealed RebM to be a member of the general acid/base-dependent O-MTases and to adopt a typical AdoMet-binding fold. Consistent with the reported ability of RebM to methylate a wide range of indolocarbazole surrogates, the model of the RebM-indolocarbazole complex implicated a predominance of nonspecific hydrophobic interactions. As the first crystal structure for a sugar O-MTase, this study may also present a template toward the future engineering of natural product MTases for combinatorial applications.

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