Molecular Basis of Mitomycin C Resistance in *Streptomyces*: Structure and Function of the MRD Protein

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Summary

Mitomycin C (MC) is a potent anticancer agent. *Streptomycyes lavendulae*, which produces MC, protects itself from the lethal effects of the drug by expressing several resistance proteins. One of them (MRD) binds MC and functions as a drug exporter. We report the crystal structure of MRD and its complex with an MC metabolite, 1,2-cis-1-hydroxy-2,7-diaminomitosene, at 1.5 Å resolution. The drug is sandwiched by π-stacking interactions of His-38 and Trp-108. MRD is a dimer. The βαβββ fold of the MRD molecule is reminiscent of methylmalonyl-CoA epimerase, bleomycin resistance proteins, glyoxalase I, and extradiol dioxygenases. The location of the binding site is identical to the ones in evolutionarily related enzymes, suggesting that the protein may have been recruited from a different metabolic pathway.

Introduction

Mitomycin C (MC, MW 331) is a highly cytotoxic, DNA-reactive antibiotic produced by *Streptomycyes lavendulae* [1]. Approved by the Food and Drug Administration as an anticancer drug in 1974, MC has been used widely and continues to be of use in the treatment of bladder, head and neck, cervical, gastric, pancreatic, and colon cancers [2–4]. In its prodrug form (Figure 1A), MC does not bind to DNA and becomes biologically active only after reductive conversion to a short-lived and highly reactive quinone methide [5–7]. The latter compound lacks the methoxide substituent, and the aziridine ring is opened, conferring high reactivity on the sp2 carbon C1. In this activated form, MC monoalkylates the 2-NH2 group of guanines in the minor groove via the C1 carbon and may form intra- and interstrand cross-links at both the C1 and C10 positions [8] (Figure 1B). Since cells halted in G2 phase will die, rapidly proliferating cells are especially vulnerable to this attack. The bioreductive activation often requires NAD(P)H as the electron donor and is enzymatically catalyzed [9]. Since many detoxification enzymes specifically bind quinones, MC can effectively "hijack" enzymes that normally neutralize free radicals in the cell. As a paradigm for a bioreductive alkylating agent, MC has attracted substantial attention [2, 10].

Reoxidation of the products of reductive metabolism of MC leads to the formation of 2,7-diaminomitosene (2,7-DAM) (nucleophilic pathway) or a mixture of 1,2-cis and 1,2-trans-1-hydroxy-2,7-diaminomitosenes (1-hydroxy-2,7-DAMs) (Figure 1C). Structurally similar to quinone methide, this compound is much less cytotoxic than MC, even though it binds DNA and may alkylate it following an additional reductive event [9, 11, 5]. Interestingly, acid hydrolysis of MC also results in a similar conversion of MC, but the predominant product is 1,2-cis-1-hydroxy-2,7-DAM, not a mixture of cis and trans isomers [12].

Two proteins in *S. lavendulae* that are products of the mcrA and mrd genes have been shown to confer resistance to MC [13–15]. MCRA (54 kDa) has a covalently bound FAD cofactor and acts by oxidizing the activated (reduced) MC before it can bind DNA [16]. In contrast, MRD, a 14 kDa, single chain protein, interacts with MC itself and functions as a component of a drug-exporting pathway [15]. Recent work indicates that MRD works in conjunction with a membrane-associated protein (MCT) to expel MC from the cell [17]. Both the mrd and mct genes are found within the MC biosynthetic gene cluster in *S. lavendulae* [18].

Clinical research suggests potential applications for proteins with functions similar to those of MRD and MCRA. A genetic therapy protocol involving the bioreductively-activated alkylating agent CB-1954 was recently approved for phase I/II studies by the FDA [19]. The protocol involves transfaction of the gene for an activating enzyme (nitroreductase) selectively to tumor cells, thereby sensitizing them to chemotherapy. Healthy somatic cells, which would only be exposed to the prodrug form of CB-1954, could therefore be spared harmful side effects. A future application of proteins similar to MRD and/or MCRA might conceivably work in an inverse manner, providing healthy cells with specific resistance to MC [20] and allowing for a much more aggressive treatment of tumors than is presently possible.

Structural characterization of the MRD resistance protein was pursued to explore the molecular basis of MC

Key words: mitomycin C; antibiotic resistance; SAD; anomalous diffraction; domain swapping; π stacking
drug binding and transport. Herein we describe the molecular architecture of the native MRD protein and the structural details of the complex of MRD with 1,2-cis-1-hydroxy-2,7-DAM, which binds to MRD with an association constant that is five times higher than that of MC. MRD is a dimer, with each monomer containing a tandem repeat of βαββ modules. Two different modes of dimer formation are observed, suggesting a domain-swapping event. The drug binding sites are located in deep clefts on the opposite sides of the molecule and formed either by the curvature of the β sheet within a monomer or, when domain swapping occurs, by two βαββ modules from adjacent monomers. The drug is sandwiched by π-stacking interactions between the protonated imidazole of His-38 and the indole ring of Trp-108.

Results and Discussion

Structure Determination and Model Quality
MRD was overexpressed in E. coli, purified, and crystallized as described in Experimental Procedures. The use of the MAD phasing technique based on SeMet was limited due to the presence of only one Met residue. Therefore, we designed a double mutant in which Leu-19 and Leu-25 were replaced by methionine residues. Crystals of the SeMet containing double mutant diffracted beyond 1.3 Å resolution (see Table 1). For experimental phasing, we used a single data set collected at the absorption peak of Se (0.979 Å) from a crystal soaked for two days in the mother liquor containing MC (see Experimental Procedures). The positions of the three methionine residues were readily determined and allowed for the calculation of preliminary phases. The initial model was created using ARP/wARP [21], which automatically built 110 out of a total of 130 residues, and refined with SHELXL [22]. Additionally, a complete data set to 1.5 Å was first collected from a crystal of the wild-type and unliganded protein.

Table 1. X-Ray Experimental Data

<table>
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<tr>
<th>Parameter</th>
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<th>Partial Ligand Occupancy</th>
<th>Full Ligand Occupancy</th>
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<td>Resolution (Å)</td>
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<td>1.3</td>
<td>1.5</td>
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<tr>
<td>Number of reflections</td>
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<td>119,032</td>
<td>66,770</td>
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<tr>
<td>Total</td>
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<tr>
<td>Unique</td>
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<tr>
<td>Redundancy</td>
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<td>99.9 (99.6)</td>
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<td>Completeness (%)</td>
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<td>6.8 (34.2)</td>
<td>4.6 (30.1)</td>
</tr>
<tr>
<td>R_f (％)</td>
<td>36.1 (3.6)</td>
<td>13.9 (3.4)</td>
<td>26.3 (4.0)</td>
</tr>
</tbody>
</table>

*The numbers in parentheses describe the relevant value for the last resolution shell.

Though the quality of the model based on the 1.3 Å SeMet dataset was good, the density presumed to correspond to the ligand was deemed unsatisfactory. Therefore, the experiment was repeated, and 1.5 Å resolution data were collected from another SeMet-containing crystal that was soaked for a longer period in the presence of MC. Subsequent refinement against these data revealed full occupancy for a bound ligand, which proved to be 1,2-cis-1-hydroxy-2,7-DAM (see below for further details of the chemistry). The final, refined model of the complex is derived from this 1.5 Å data set.

In all three crystal structures, a single monomer occupies the asymmetric unit of the C2 unit cell, and two monomers form a close association with the resultant 2-fold axis of the MRD dimer corresponding to the crystallographic dyad. This is consistent with our gel filtration results (data not shown), which indicate that a dimer is the predominant species in solution. For all three refined models, each monomer contains residues 3–130, inclusive, with the region containing residues 65–71 being comparatively less well ordered. Residues in this region were not included until final refinement, for reasons explained below. There are 153, 161, and 158 ordered water molecules included in the final models for the ligand-bound, apo form, and 1.3 Å resolution structures, respectively. In the case of the complex structure, a molecule of 1,2-cis-1-hydroxy-2,7-DAM was modeled into residual positive difference electron density and included late in refinement. The known geometry of the quinone ring was used as a restraint, while the remaining moieties were restrained using standard bond lengths and angles.

The quality of the models, as judged by stereochemical criteria, is high (see Table 2), and PROCHECK [23] analysis indicates that approximately 95% of the residues in the final models are in the most favored regions with no outliers.
Structural Analysis of the Mitomycin C Resistance Protein

Table 2. Refinement Details

<table>
<thead>
<tr>
<th></th>
<th>Partial Ligand Occupancy</th>
<th>Full Ligand Occupancy</th>
<th>Apo-MRD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution used in final refinement (Å)</td>
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<td>10–1.5</td>
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<td>13.6/20.9</td>
<td>16.8/23.4</td>
</tr>
<tr>
<td>Rmsd from target bond lengths (Å)/bond angles (°)</td>
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<td>0.018/2.14</td>
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<td>Rmsd from &lt;B&gt; (Å&lt;sup&gt;2&lt;/sup&gt;) overall</td>
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</tr>
<tr>
<td></td>
<td>Waters</td>
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</tr>
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</table>

<sup>a</sup>R<sub>crys</sub> = Σ|F<sub>c</sub> – F<sub>s</sub>|/Σ|F<sub>c</sub>|, where F<sub>c</sub> is the calculated structure factor.

MRD Is a Member of a Diverse Family of βαββ Proteins

MRD is an oblong dimer with dimensions of roughly 50 × 40 × 30 Å (Figure 2B). Each monomer is made up of an eight-stranded β sheet, with each sheet comprised of two βαββ motifs (domains) packed edge to edge, so that the first strands of the two modules are associated in an antiparallel manner. The basic topology in each domain is a four-stranded β sheet (defined as +1, +4, −3, and +2) with an α helix connecting strands 1 and 2 (Figure 2A). Domain A consists of residues 3–53, and domain B consists of residues 74–130, while residues 54–73 form the connecting region, including a short α helix. In spite of lack of identifiable sequence similarity between the two domains, their tertiary structures are very close. A superposition of the two modules in MRD yields 38 pairs of Cα atoms with a root-mean-square (rms) difference of 1.6 Å.

Tandem βαββ motifs have been observed in several other proteins with diverse functions. They originally included the bleomycin resistance protein from *Streptomyces lincolnensis* (ShBle) [24], two extradiol dioxygenases [25, 26], and glyoxalase I [27]. These structures were quickly recognized to be representative of a larger family [28]. More recently, additional members were described: the bleomycin resistance proteins found in *Streptomyces verticillus* (BLMA) [29] and on transposon Tn5 in *E. coli* (BLMT) [30], a methylmalonyl-coenzyme A epimerase (MMCE) from *P. shermanii* [31], and several more dioxygenases and glyoxalases [32–35].

Of particular interest is the structural similarity between MRD and the bleomycin resistance proteins (BRPs). As with MC, bleomycin is an antibiotic and DNA cleaving agent but is chemically unrelated. However, there is some identifiable, albeit low, amino acid se-

Figure 2. The Structure of MRD Protein
(A) Topology of the MRD monomer. Domain A, pink; domain B, red.
(B) Stereoview of the Cα tracing of the MRD dimer.
Structure 936

Figure 3. Structure-Based Sequence Alignment of MRD with Bleomycin Resistance Proteins ShBle (1BYL), BLMT (1ECS), and BLMA (1QTO)

The two key residues involved in sequestering the drug are boxed in red; the five invariant residues in the sequences are boxed in green.

The family of proteins to which MRD belongs is recognized for a history of gene duplication and domain-swapping events [28, 31, 36]. The original gene duplication event led to the $\beta\alpha\beta\beta$ tandem module, which appears to require dimerization for stability. Owing to the nature of the dimer, in which the connecting linkers are close together, two alternative modes of dimerization are possible. Interestingly, while MMCE conforms to one of these (form I), BRPs and human glyoxalase exemplify the other (form II), and the two groups were contrasted in literature as evidence of evolutionary 3D domain swapping [28]. Further duplication is observed in dioxygenases, where two tandems are covalently linked to form a sequential 4-fold repeat of the $\beta\alpha\beta\beta$ module. The MRD structures, on the other hand, appear to suggest that the domain-swapping events may be dynamic, so that the two forms may coexist in solution, while crystallization selectively isolates either one or the other. Whether this is a consistent feature of other proteins in the family remains to be seen.

The Bound MC Derivative Is 1,2-cis-1-Hydroxy-2,7-DAM

MRD crystals turn pink when soaked for a few days in mother liquor solution containing crystalline MC. Because MC is purple, with a visible absorbance spectrum showing a single peak at 360 nm, the pink color suggested the appearance of a compound closely related to 2,7-diaminomitosene, with a characteristic absorbance peak at 313 nm. The initial 1.3 Å data set, derived from crystals soaked for two days, yielded a region of density suggestive of a bound ligand, albeit of poor quality. The 1.5 Å data set, obtained from a crystal soaked for four days, yielded very well-defined electron density for the bound drug. We noted that there was no electron density associated with the methoxy substituent at C9a of MC, and the region corresponding to C9 carbon clearly showed the flattened sp$^2$ hybridization, with the substitu-
Figure 4. The Two Topological Forms of the MRD Dimer
Form I and form II are on the left, close-ups of their respective “crossover” region are in the center, and schematic diagrams of the β strands (represented perpendicular to the page) are on the right. In form I, each β sheet is created by a single monomer (A connected to B), while, in form II, each sheet is composed of the A domain from one monomer and the B domain of the other (A connected to B). For each protein, one monomer is shown with its first domain pink and its second domain red (the convention used in Figure 2), while the other monomer is shown with its first domain yellow and its second domain gold. The electron density shown is from omit maps, with the three relevant residues removed for several rounds of least-squares refinement.

...unt in the plane of the quinone, rather than the tetrahe-
dral sp² geometry associated with MC (Figure 5A). Fur-
ther, there was clear evidence (as judged from the electron density) of the opening of the aziridine ring with two substituents in positions 1 and 2 in cis configuration. All these details are consistent with 1,2-cis-1-hydroxy-
2,7-DAM, and not MC, being bound to MRD. For final
refinement a set of model constraints, based on the
known crystal structure of MC, was used [37].

Since 1,2-cis-1-hydroxy-2,7-DAM is a reaction prod-uct derived from either reduction or acid hydrolysis of
MC, we decided to determine whether MRD has cata-
lytic properties under the conditions used to generate
crystals. We monitored the stability of MC in solution by
measuring absorption spectra in the visible wavelength
range. The hydrolysis of MC to 1-hydroxy-2,7-DAM is
accompanied by a change of spectrum with excellent
isosbestic points at 330 and 240 nm, indicating that no
intermediates are involved [12], and the reaction is easily
followed by spectrophotometric means. Our data (data
not shown) indicate that MC is stable in 100 mM MES
buffer at pH 6.0 in both the presence and absence of
MRD. However, the addition of 65% saturated ammo-
nium sulfate results in the hydrolysis of MC to 1,2-cis-
1-hydroxy-2,7-DAM over a period of hours to days, again
with and without addition of MRD. We conclude that
under the conditions of our experiments, MRD is not
involved in the hydrolytic transformation of MC, which
occurs in solution due to general acid/base catalysis.

The structural analysis of MRD shows that its drug
binding site is located within a deep cleft formed by the
curved eight-stranded β sheet, with the flat hydroqui-
one moiety of 1-hydroxy-2,7-DAM packing tightly into
a “slot” created by the side chains of residues His-38
and Trp-108 (see below for further details). The presence
of a tryptophane in the binding site allowed us to mea-
sure the dissociation constants (K_d) for MC and 1-hydroxy-
2,7-DAM using fluorimetric titration. The dissociation
constant for MC determined in this way is 31 μM, while
that for 1-hydroxy-2,7-DAM shows tighter binding—6.3
μM. This shows that the integrity of the aziridine ring
and the methoxide group of MC are not required for
efficient binding and that MRD is capable of binding
both MC and its immediate reaction product.

The Drug Binding Site
The symmetry-related binding pockets are located on
opposite sides of the dimer. In form I, each binding
cavity is created by the bent, or “cup”, structure of the
eight-stranded β sheet of each monomer. In form II, the

...
Figure 5. The Binding of 1,2-cis-1-Hydroxy-2,7-DAM to MRD
(A) Electron density and a model of the bound 1,2-cis-1-hydroxy-2,7-DAM molecule. The density shown was generated using an omit map, with ligand model removed before several rounds of refinement.
(B) Two views of the bound form of the MRD dimer, again in form II, with helices in red and β sheets in light blue. The DAM molecule is depicted in yellow.

β sheet is created from four strands from each monomer, and the binding pocket is therefore also composed partly from one monomer and partly from the other (Figure 5B). Regardless of the topological differences between the two forms, the chemistry of the binding site remains the same.

The most noticeable feature of the substrate binding region is the outer rim, where the flat hydroquinone moiety of DAM packs tightly into a slot created by the imidazole of His-38 and the indole ring of Trp-108. It is interesting to note that His-38 remains protonated and charged, due to two interactions with carboxylates, one with Asp-52 through Nε1 and one with Glu 40 via Nε2. Both are short H bonds with 2.6 Å distances. The imidazole (His-38) and the five-membered portion of the indole (Trp-108) face each other, with their centers approximating a common axis for both rings. The hydroquinone moiety is sandwiched between these two rings, so that the C8a carbon packs between the centers of the rings. A nearly straight line can be drawn from the Cε1 carbon of the histidine through the C8a carbon of the quinone to the Cγ carbon of the tryptophan. The total separation is 7.0 Å, with the C8a situated almost precisely in between, at a distance of 3.5 Å from the other two atoms (Figure 6A).

The region of the DAM molecule corresponding to the location of the aziridine ring in MC makes contact with residues in the interior of the protein, while the opposite side is exposed to bulk solvent. The C9 substituent is held in place by a set of hydrogen bond interactions, notably the terminal N14 nitrogen with Tyr-112 and the terminal O13 oxygen with Gln-110, and by two water molecules within the cavity. The specific binding of this amidoacetate group appears important because the dissociation constant for MC of the Y112F mutant, in which one of the hydrogen bonds is removed, is 0.4 mM, an order of magnitude higher than that of the wild-type protein. There is also an H bond network involving the hydroxy oxygen (designated O0 in the model) and the amino nitrogen N1 (which is an aziridine nitrogen in MC). This network involves a water molecule that forms bonds with both the O0 oxygen and the N1 nitrogen. Two other residues, Tyr-60 and Asp-52, each form a hydrogen bond with the N1 nitrogen as well. The negative charge on Asp-52 is not critical because the D52N mutant binds MC with affinity virtually identical to the wild-type protein.

The overall arrangement of residues in wild-type unliganded MRD is not significantly different from the drug-bound form. The distance between His-38 and Trp-108 is slightly larger in the native form, so the cleft between them appears to narrow when packed with the DAM quinone. The space occupied by the ligand in the complex is filled by approximately eight ordered water molecules in the unliganded form of the protein. The overall arrangement of the hydrogen bonding network, particularly one involving Asp-52 and Tyr-60, can be seen in Figure 6B. The bound 1,2-cis-1-hydroxy-2,7-DAM does not induce any significant structural changes in the MRD protein.

Comparison with BRPs
As already pointed out, MRD shares its overall tertiary and quaternary structure with BRPs, which also have a
pair of symmetric cavities that serve as binding sites, but there are substantial differences between the two target drugs. Bleomycin, the ligand for BRPs, is a far larger molecule than MC and chemically unrelated. It contains several chemical groups, including bithiazole, methylvalerate threonine linker, and metal binding and sugar moieties. Recently, a crystallographic study revealed the structural details of the BLMT-Bm complex [30]. The linker, metal binding, and sugar groups of Bm interact with numerous amino acids within a shallow surface groove, but the bithiazole moiety, made up of two five-membered rings, is sequestered between two tryptophanes, i.e., Trp-99 and Trp-35, which correspond to Trp-108 and His-38 of MRD. The loops containing these crucial residues have virtually identical stereoch- emistries in the two proteins, although the details of the interaction with ligand vary (Figure 7). Trp-108 of BLMT has the indole ring rotated nearly 180° around \( \chi_2 \), as compared to MRD, and Trp-35 has the five-mem- bered ring nearly overlapping with the imidazole of His-38 when the two models are overlapped. The distance between the two parallel indoles is about 7 Å. The first thioazole group of Bm interacts only with Trp-108, while the second thiozole is sequestered by both indoles. BLMT binds Bm with a dissociation constant of 32 nM, but it is not known whether bithiazole binds on its own and with what affinity. Thus, even though the nature of the ligand is very different, the mode of drug binding by the bleomycin resistance proteins and MRD is very similar.

**Biological Implications**

The study reported in this paper describes the high-resolution crystallographic study of the MRD resistance protein in complex with a derivative of the cancer drug mitomycin C. The MRD dimer is believed to be part of a drug transport system that shuttles the MC molecule out of the drug-producing organism *Streptomyces lavendulae*. The protein is a member of a larger family of enzymes and drug binding molecules characterized by a conserved \( \beta_0\beta\beta\beta \) motif as a principle module. The quinone moiety of the bound 1,2-cis-1-hydroxy-2,7-DAM is sandwiched between the imidazole of His-38 and the indole of Trp-108. Affinity measurements show that both mitomycin C and 1-hydroxy-2,7-DAM bind with similar dissociation constants (in the \( \mu M \) range). The binding mode is similar to that observed for the bleomycin resistance protein, where a bithiazole moiety is sequestered between two tryptophan residues that occupy sites equivalent to those of Trp-108 and His-38 in MRD. This suggests that the \( \beta_0\beta\beta\beta \) proteins are promiscuous in their interactions with ligands and could be engineered to bind a diverse set of planar molecules of biological importance.
Experimental Procedures

Expression of MRD in E. coli

The mrd gene was subcloned into an expression vector used to create a GST fusion protein with an rTEV proteolytic cleavage site (pGST-Parallel1) [38]. Aggregation difficulties with the MRD-GST fusion protein led us to test samples using size exclusion chromatography. This provided early evidence that MRD was a dimer and that higher-order oligomers of MRD-GST were responsible for aggregation. The mrd gene was then subcloned into a His6-tag expression vector (pHis Parallel1) [38]. The sequence-verified plasmid was transformed into E. coli BL21(DE3) (Novagen). The cells were grown at 37°C in 1 liter of LB broth containing 100 µg/ml ampicillin to an OD600 of 0.6–0.8, and expression was initiated by the addition of IPTG to a final concentration of 1 mM. The culture was grown for an additional 3 hr at 37°C. The cells were harvested by centrifugation at 8,000 × g for 10 min at 4°C. The pellet was resuspended in ice-cold His buffer (5 mM; 300 mM NaCl and 50 mM Tris-HCl [pH 8.5]) and sonicated on ice using five 30 s bursts with a 5 min cooling period between each burst. The cellular debris was pelleted by centrifugation at 11,500 × g for 30 min at 4°C, and supernatant was used for further purification of MRD.

Purification of MRD

Purification of His6-tagged MRD was carried out by standard methods with slight modifications. The supernatant was passed through a column containing 5 ml of preequilibrated (His buffer) Ni2+-NTA agarose to bind the His6-tagged MRD, and the resin was washed with 2 liters of His buffer at 4°C. The bound protein was recovered with elution buffer containing 250 mM imidazole (pH 8.5). The His6-tag was cleaved with 1 µl of rTEV protease ( Gibco) per milliliter of sample for 48 hr at room temperature. A 10,000 MWCO Slide-a-Lyser cassette (Pierce) was used for dialysis against 4 liters of His buffer to remove imidazole and the cut His6-tag. The diazylated solution was passed through a column containing 5 ml of Ni2+-NTA agarose to remove both uncleaved protein and rTEV protease. The protein was concentrated to ~3 mg/ml by using an Amicon 8050 ultrafiltration unit with a YM 10 membrane (10,000 MWCO; Millipore).

Mutagenesis and Preparation of SeMet Samples

The L19M and L25M mutations used to introduce additional methionine residues for phasing purposes, as well as the D52N and Y112F mutations designed to probe the specificity of the binding site, were introduced into the MRD gene using the QuickChange protocol. All constructs were verified by direct sequencing prior to further use. To obtain SeMet samples, the auxotrophic strain B834 was transformed with the pHIS plasmid. Cultures were grown in LeMaster medium, with selenomethionine replacing methionine. The expression protocol paralleled that of the native protein described above, with the important exception that 1 day was required for the culture to reach the necessary optical density, instead of several hours. The purification protocol was likewise identical, with the exception that the rTEV cleavage reaction took nearly a week to run to completion, instead of two days.

Binding Assays

Dissociation constants (Kd) were determined by fluorimetric titration of MRD wild-type and its two mutant forms (D52N and Y112F) with increasing concentrations of drug (MC or DAM). To determine the dissociation constant, increasing amounts of the drug were added to a constant concentration of the MRD. In each case, eight to ten data points were collected. Experiments were done in duplicate at 20°C using an FP-750 spectrofluorometer (Jasco) under the following conditions: λexcitation = 280 nm, λemission = 342 nm, and excitation and emission slit width = 5 nm. MC or 1-hydroxy-DAM (2 µM–0.7 mM) were added to 1200 µl of 1.8 µM MRD in 100 mM MES (pH 6.0). The residual protein fluorescence was measured after 100 min incubation. The dissociation constants were determined as described previously [39]. The protein concentration was estimated using the molar absorbance coefficient at 8,000 M−1 cm−1 for MRD WT and MRD D52N and at 10,189 M−1 cm−1 for MRD Y112F, calculated from the number of Trp and Tyr residues in the MRD molecule [40]. The MC concentration was estimated following the use of the molar absorbance coefficient at 21,840 M−1 cm−1. The DAM concentration was determined using the molar absorbance coefficient at 10,189 M−1 cm−1, determined by the total acidic hydrolysis of the known amount of MC.

Crystallization and Sample Preparation

Crystallization was accomplished using protein at 3 mg/ml concentration in 55% ammonium sulfate and 100 mM MES at pH 6.0. Native crystals were small and frequently twinned. Addition of β-octylglucoside to a final concentration of 0.05% slowed down crystal growth and reduced twinning. SeMet crystals grew in similar conditions, with the exception of a small increase in ammonium sulfate (65%). The SeMet crystals grew more slowly (3–4 weeks) and were typically single and larger, with maximal dimensions of 0.25 × 0.15 × 0.15 mm. SeMet crystals were soaked in mother liquor with increased ammonium sulfate content (100 mM MES and 75% ammonium sulfate) containing crystalline mitomycin C and NADH at a concentration of approximately 1–2 mM each. NADH was used because of previously published data, which suggested that MC binding by MRD is NADH dependent [16]. However, our present data show that, in the presence of NADH, MRD binds MC with a dissociation constant of 24.1 M−1, i.e., within experimental error the same as in its absence. Soaks without NADH produced pink MRD crystals in comparable time. Native and soaked SeMet crystals were flash frozen using mother liquor solution with high carbons of the two sequestering amino acids superposed on each other.

Figure 7. The Comparison of the BLMT and MRD Binding Sites

The 1,2-cis-1-hydroxy-2,7-DAM molecule and the bithiazole of BM are shown with the α carbons of the two sequestering amino acids superposed on each other.
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Accession Numbers

Atomic coordinates were deposited in the Protein Data Bank under accession codes 1KMZ (unliganded MRD) and 1KLL (MRD with bound 1,2-cis-1-hydroxy-2,7-diaminomitoseno).