Structures of BmrR-Drug Complexes Reveal a Rigid Multidrug Binding Pocket and Transcription Activation through Tyrosine Expulsion

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BmrR is a member of the MerR family and a multidrug binding transcription factor that up-regulates the expression of the bmr multidrug efflux transporter gene in response to myriad lipophilic cationic compounds. The structural mechanism by which BmrR binds these chemically and structurally different drugs and subsequently activates transcription is poorly understood. Here, we describe the crystal structures of BmrR bound to rhodamine 6G (R6G) or berberine (Ber) and cognate DNA. These structures reveal each drug stacks against multiple aromatic residues with their positive charges most proximal to the carboxylate group of Glu-253 and that, unlike other multidrug binding pockets, that of BmrR is rigid. Substitution of Glu-253 with either alanine (E253A) or glutamine (E253Q) results in unpredictable binding affinities for R6G, Ber, and tetraphenylphosphonium. Moreover, these drug binding studies reveal that the negative charge of Glu-253 is not important for high affinity binding to Ber and tetraphenylphosphonium but plays a more significant, but unpredictable, role in R6G binding. In vitro transcription data show that E253A and E253Q are constitutively active, and structures of the drug-free E253A-DNA and E253Q-DNA complexes support a transcription activation mechanism requiring the expulsion of Tyr-152 from the multidrug binding pocket. In sum, these data delineate the mechanism by which BmrR binds lipophilic, monovalent cationic compounds and suggest the importance of the redundant negative electrostatic nature of this rigid drug binding pocket that can be used to discriminate against molecules that are not substrates of the Bmr multidrug efflux pump.

Multidrug-resistant bacteria have become an extreme threat to human and animal health (1). One mechanism of multidrug resistance is the active extrusion of chemically and structurally dissimilar drugs by multidrug efflux transporters (2, 3). These pumps are often regulated by transcription factors that respond to the same toxins and biocides that serve as substrates for the transporters (2–5). Thus, these transcription regulators are also multidrug-binding proteins, and the delineation of their multidrug binding mechanisms provides high resolution insight into those of the efflux pumps.

To date, structures of multidrug binding transcription factors in complex with multiple drugs have been limited to QacR and TtgR, both of which belong to the TetR family (6–8). The multidrug binding pocket of QacR is formed after the expulsion of two tyrosine residues from the protein core to expose a voluminous binding site that is composed of aromatic and negatively charged residues and overlapping “minipockets,” which are utilized selectively by monovalent and bivalent cationic drugs (8). The multidrug binding pocket of TtgR, which binds neutral and negatively charged toxins, has two overlapping sites, a general site, which is highly hydrophobic, and a second, more polar site, which includes a basic residue that is key to the high affinity binding of the plant antimicrobial phloretin (6). However, a serious limitation to our understanding of multidrug binding and its resulting gene regulation is the dearth of structural and biochemical data on multidrug binding transcription regulators from other families.

BmrR, the bacterial multidrug resistance regulator from Bacillus subtilis, activates transcription of the bmr multidrug transporter gene by binding to a plethora of structurally dissimilar lipophilic, monovalent cationic compounds, which are also the substrates of Bmr (9, 10). BmrR belongs to the MerR family, which activates transcription from noncanonical $\sigma^{70}$ and $\sigma^A$-regulated promoters in response to a variety of stresses (11–13). These promoters typically contain 19- or 20-bp spacers between the –10 and –35 promoter elements in contrast to the canonical 17-bp spacing found in most $\sigma^{70}$ or $\sigma^A$ responsive promoters (13–15). The 19-bp spacer places these promoter hexamers ~72° out of phase on the DNA helical axis, thereby preventing productive open complex formation by RNA polymerase (16–19).

In the absence of the appropriate signal, nearly all MerR family members bind their promoters with high affinity but do not activate. Drug binding to the BmrR-promoter complex enables productive interaction of RNA polymerase with the promoter (9).
Structures of BmrR-Drug Complexes

The structure of BmrR in complex with a 22-bp oligodeoxyribonucleotide, encompassing most of the bmr promoter, and the "drug" tetrphenylphosphonium (TPP+)2 has been solved (20). The structure revealed an N-terminal winged helix DNA binding domain and a C-terminal drug binding domain that are linked by a long antiparallel coiled-coil. The structure also revealed kinking and under-twisting of the DNA, which shrinks the helix by ~5 Å and reconfigures the ~10 and ~35 elements to resemble their positions in canonical α70/αE responsive promoters. The universality of DNA under-twisting, shrinking, and bending as the transcription activation mechanism by MerR family members is supported by the structures of MtaN and SoxR-DNA complexes (21, 22).

Despite our knowledge of the structure of BmrR in its transcription activated conformation, the molecular mechanisms by which this protein binds drugs and activates transcription of the bmr gene remain unclear. Crystal structures of the C-terminal drug binding domain of BmrR (BRC) both with and without TPP+ provided some initial insight and implicated glutamate residue Glu-253 at the base of the multidrug binding pocket in the neutralization of cationic drugs (23). In the absence of drug the carboxylate side chain of Glu-253 interacts with three tyrosine residues, Tyr-152, Tyr-187, and Tyr-229, which “neutralize” this buried negative charge. Tyr-152 resides at the N terminus of a small α helix that covers the drug binding pocket, which upon drug binding moves away from the pocket to accommodate drug. Mutational studies on the BRC support the functional importance of Glu-253, as its substitution with alanine or glutamine had a noticeable effect on ligand binding affinity (10). Charge neutralization of drugs by oppositely charged residues in the drug binding pockets of TetR family members QacR, TtgR, and AcrR has been postulated or shown to be important for drug binding and induction (6–8, 24–26). Regardless, the role of Glu-253 in drug binding and transcription activation in the context of full-length BmrR is unknown, as an additional acidic residue, Asp-47, from the DNA binding domain of the other subunit, was absent in the studies on the BRC yet found proximal to TPP+ in the BmrR-TPP+ -DNA complex (20, 23).

Given the dearth of structural knowledge of the mechanisms of multidrug binding by transcription factors outside the TetR family, structural studies on BmrR bound to either rhodamine 6G (R6G) or berberine (Ber), and site-directed mutants in which Glu-253 was replaced by either alanine (E253A) or glutamine (E253Q) were undertaken. In parallel the effects of these mutations on drug binding affinity and transcription activation were determined. These studies define the structural mechanism of multidrug binding, which parallels other multidrug binding transcription regulators yet differs significantly in key aspects.

EXPERIMENTAL PROCEDURES

In Vivo and in Vitro Transcription Activation—The E253A and E253Q mutations were introduced into the bmrR gene of wild type B. subtilis strain BD170 by transformation with a PCR product containing each mutation. The PCR product, which was generated by hybrid PCR, included flanking sequences of 1.0 and 1.4 kilobases upstream and downstream of the mutation, respectively. To allow quick selection of the transformants, DNA from a B. subtilis strain containing the chloramphenicol resistance gene Cm16 inserted downstream of bmrR was used as a template such that the downstream primer incorporated this resistance marker into the PCR product. BD170 cells were transformed with the purified hybrid PCR product containing either the E253A or the E253Q mutation and selected for chloramphenicol resistance at 4 μg/ml. Transformants containing the E253A or E253Q mutation were identified by direct sequencing and compared with the transformants with wild type bmrR for their sensitivity to ethidium bromide by a broth microdilution assay in 96-well plates (9).

Cloning, expression, and purification of mutant BmrR-His6 proteins were performed as described previously for wild type BmrR (10). Purification of B. subtilis RNA polymerase and in vitro transcription assays were also performed as described previously (9, 10). Briefly, a 221-bp PCR fragment containing the bmr promoter was used as a template to generate a 98-nucleotide runoff mRNA transcript. Transcription reactions (25-μl reaction volume) contained 0.3 pmol of bmr template DNA, 0.3 pmol of rpsD promoter DNA, and 1.2 pmol of wild type, E253A, or E253Q BmrR-His6 protein. 50 pmol of R6G, 100 pmol of Ber, or 1 nmol of TPP+ was added during the preincubation reaction where indicated. The runoff transcripts labeled with [α-32P]UTP were separated by electrophoresis on a 6% polyacrylamide gel containing 7 M urea and visualized by phosphorimaging (GE Healthcare), and bands were quantitated using the ImageQuant software (GE Healthcare).

Drug Binding Assays Using Fluorescence Polarization—Fluorescence polarization-based BmrR-drug binding experiments were done with a PanVera Beacon fluorescence polarization system (Invitrogen) utilizing the intrinsic fluorescence of R6G and the increase in polarization that is observed upon the binding of this small compound (molecular weight = 479 Da) to the ~65-kDa protein dimer. Binding was assayed in a 1-ml volume at 25 °C. Each binding experiment contained 1 nM R6G in binding buffer (25 mM sodium phosphate, pH 7.3, 150 mM NaCl, 1 mM EDTA, and 5% glycerol). In one set of control experiments, DNA corresponding to the bmr promoter in the absence of any protein was titrated with R6G. After each addition of protein-DNA complex or protein alone, the samples were incubated for 30 s before a measurement was taken. The millipolarization (P10–3, where P is polarization) at each titration point represents the average of eight measurements integrated over 6 s. Samples were excited at 490 nm, and emission was measured at 560 nm. Each binding isotherm was analyzed by curve fitting with Kaleidograph using the equation P = [Pbound - Pfree] (protein-R6G)/(Kd + [protein-R6G]) + Pfree, where P is the polarization measured at a given concentration of protein, Pfree is the initial polarization of the free R6G, and Pbound is the maximum polarization of specifically bound R6G (26). Nonlinear least squares analysis was used to determine Pbound and Kd.

Drug Binding Assays Using Isothermal Titration Calorimetry—Isothermal titration calorimetry (ITC) measurements were made using a VP-Microcalorimeter (MicroCal, Northampton,
MA) at 35 °C. After our standard purification procedure (20), the protein was further purified by gel filtration to remove aggregates and contaminating DNA. The protein was eluted in 25 mM sodium phosphate, pH 7.3, 150 mM NaCl, 1 mM EDTA, and 5% glycerol. Stock solutions of berberine and R6G were prepared as saturated solutions in dimethyl sulfoxide (DMSO) then diluted with the gel filtration buffer to a final concentration of between 0.5 and 1 mM, which corresponded to a final concentration of DMSO of 1–2%. An equivalent amount of DMSO was added to the protein sample. TPP + was prepared at a concentration of 5 mM in the gel filtration buffer. Heats of dilution were measured by injecting ligands into the buffer and were subtracted from the raw data. All binding isotherms were fit to a one-site model using the ORIGIN software provided by MicroCal.

Crystallization, Structure Determination, and Refinement of the E253A-DNA and E253Q-DNA Complexes—The mutant BmrR proteins were purified and resolubilized with DNA as described previously (20). As with wild type BmrR, the same 22-base pair duplex oligodeoxynucleotide containing the bmr promoter sequence 5′-AGACTCTCCCCTAGGAGGAGGTC-3′ and a 5′ overhanging adenosine was used for crystallization with the exception that the mutant proteins were crystallized under different conditions. The E253A-DNA and E253Q-DNA crystals were grown by the hanging drop vapor diffusion method with a reservoir solution containing 0.85M E253Q-DNA crystals were grown by the hanging drop vapor diffusion method. Four concentrations of reservoir solution and 1 M trisodium citrate, 0.1 M imidazole, pH 8.0. Four μl of the protein-DNA complex (8 mg/ml) was mixed with 5 μl of the reservoir solution and 1 μl of 40% trifluoroethanol. Long tetragonal rods grew in 1–2 days to maximum dimensions of 0.2 × 0.2 × 0.5 mm. Crystals were cryoprotected in a solution containing 1 M trisodium citrate, 0.1 M imidazole, pH 8.0, and 30% glycerol and flash-frozen in a cryocooled nitrogen stream at 100 K.

X-ray intensity data were collected at the Stanford Synchrotron Radiation Laboratory beamline 9-1 for both the E253Q-DNA and E253A-DNA complexes. Both mutant crystals were isomorphous to the wild type BmrR-DNA structure and took the tetragonal space group P4322. The intensity data were processed with MOSFLM (27) and scaled with SCALA as implemented in CCP4 (28). The wtBmrR-DNA complex was used as a starting model for refinement using CNS (29).

RESULTS AND DISCUSSION

Selective Importance of Glu-253 in Drug Binding by BmrR—Previous studies on the BRC indicated that the buried residue Glu-253 was important for binding drugs; mutation of Glu-253 to alanine decreased the binding affinity by more than 10-fold for all but one of the six drugs tested (10, 23). Subsequent structural studies revealed that the BRC does not recapitulate the complete drug binding pocket of the full-length BmrR dimer (supplemental Fig. S1) (20). Therefore, we determined the drug binding affinities of BmrR and the E253A and E253Q mutants.

To do so, the intrinsic insolubility of BmrR, which precluded ITC experiments, was alleviated by creating the BmrR R275E mutant. This protein retains wild type drug binding affinity and in vitro transcription activation (Fig. 1A and supplemental S2). Therefore, all Glu-253 mutations also contained the R275E change, and we will refer to the R275E protein as wtBmrR.

Thermodynamic parameters and binding affinities were determined for R6G, Ber, and TPP + by ITC, and all drugs bound with a stoichiometry of 1 drug per BmrR dimer (Fig. 1B, Table 1). Because of unmeasurable binding heats, R6G binding affinity for the E253Q mutant was determined by a fluorescence polarization-based assay that utilized the intrinsic fluorescence of R6G (supplemental Fig. S2). The binding affinities of wt-BmrR for R6G measured by fluorescence polarization were within 2-fold that of those measured by ITC (K d ~ 0.75 μM) confirming the utility of both methods for determining binding constants (Table 1, supplemental Fig. S2). Different from the BRC, mutation of Glu-253 to either glutamine or alanine had little effect on the binding affinity for either Ber or TPP + but clearly had a larger, more complex effect on R6G binding (Table 1). The dissociation constant (K d) for R6G binding to the E253A mutant was ~8-fold higher than that of wtBmrR. Strikingly, the K d for E253Q binding to R6G was 34.1 μM, an ~87-fold reduction in binding affinity (Table 1). Thus, in the context of the full-length protein, a negative charge on residue 253 is unimportant for binding TPP + or Ber but contributes significantly to R6G binding.

Structures of BmrR-R6G-DNA and BmrR-Ber-DNA Ternary Complexes—To understand the unexpected results of the drug binding experiments as well as to delineate the atomic details of BmrR-R6G and BmrR-Ber binding, we determined their crystal structures in complex with cognate DNA (Fig. 2). To do so, a suitable cryoprotectant was developed for crystals of the previ-
ously described BmrR-TPP<sup>+</sup>-DNA complex (20) and resulted in an increased resolution from ~3.0 to 2.4 Å (21). Unexpectedly, in lieu of TPP<sup>+</sup>, the multidrug binding pocket contained four imidazole molecules, three of which form the corners of an isosceles triangle with the fourth sitting in the triangle toward the bottom (Fig. 2A). On hindsight, imidazole binding is not surprising, as this molecule is aromatic and partially positive at pH ≤ 6.5 and at a concentration of ~1 M, used for crystallization, should compete well against all BmrR ligands. Indeed, imidazole decreases BmrR binding to R6G by 22-fold at concentrations as low as 125 mM (data not shown). Thus, TPP<sup>+</sup> with a $K_d$ ~ 74 µM was out-competed by ~1 M imidazole. To avoid this complication, crystals of all other wtBmrR-drug-DNA complexes were grown without imidazole followed by soaking in saturating amounts of drugs. The resulting structures of the ternary complexes of wtBmrR-R6G-DNA and wt-

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**FIGURE 1.** Transcription activation and drug binding by wtBmrR and BmrR mutants. A, in vitro transcription activity of BmrR and the R275E (WT), E253A, and E253Q mutant proteins. Lane 1 contains no BmrR protein, lanes 2–17 contain the BmrR protein as indicated on the top of the gel. Lanes labeled — contain no drug, R lanes contain R6G, B lanes contain berberine, and the T lane contains TPP<sup>+</sup>. The top band on the gel corresponds to the 98 nucleotide bmr transcript. The bottom band corresponds to the 71-nucleotide transcript generated from the constitutive rpsD (ribosomal protein S4) promoter, which was used as a loading control. B, ITC measurements of drug binding to wtBmrR(R275E). ITC experiments were performed at 35 °C as described under “Experimental Procedures.” The upper panels show the heat changes (µcal/s) for titration of a series of 10-µl aliquots of 1 mM Ber, 500 µM R6G, and 5 mM TPP into a solution of 35–70 µM BmrR dimer. The molar ratio (x axis) is the ratio of drug to protein dimer at each titration point. The lower panels show the integrated peak areas fit with ORIGIN using a one-site binding model.
BmrR-Ber-DNA were solved to 2.6 and 2.7 Å resolution, respectively (Table 2).

As anticipated, the overall structure of the wtBmrR-R6G-DNA complex is identical to the imidazole-grown wtBmrR-DNA structure with an r.m.s.d. over all Cα atoms of 0.48 Å. Notably, the side chains in the drug binding pocket are in identical positions to those of the BmrR-imidazole-DNA structure.

The R6G molecule binds near Glu-253, Tyr-187, and Tyr-229 and occupies the space that was filled by the four imidazole molecules in the wtBmrR-DNA structure (Figs. 2, B and D, and 3B). R6G interacts with multiple aromatic and hydrophobic residues including, Ile-51, Val-147, Leu-148, Tyr-152, Ile-182, Phe-224, Phe-226, Ile-255, and Tyr-268, which provide considerable binding strength (Fig. 2B). The Oe2 carboxylate oxygen...
of Glu-253 is 3.9 Å from the positive charge on the ethyl ammonium nitrogen of R6G, thereby "neutralizing" both buried charges. Also potentially contributing to drug charge neutralization is its charge-dipole interaction with the hydroxyl group of Tyr-152. In addition, the phenyl ring of Tyr-229 stacks on top of the other ethyl ammonium nitrogen further from Glu-253, providing a favorable π-cation interaction. Unlike QacR-R6G binding, no hydrogen bonds are made between this drug and BmrR.

The overall structure of the wtBmrR-Ber-DNA complex is also identical to the BmrR-imidazole-DNA complex with an r.m.s.d. over all Cα atoms of 0.56 Å. Berberine is an alkaloid secreted from the roots of plants and may be a natural activator of BmrR given that B. subtilis is a soil bacterium that is likely to come into contact with plant antimicrobials. Thus, the BmrR-Ber-DNA ternary complex represents a physiologically relevant multidrug binding protein-coactivator-DNA complex. Berberine binds in the drug pocket in an orientation similar to R6G and is surrounded by the same aromatic and hydrophobic residues (Figs. 2C and 3A). By contrast to R6G, the positive charge on the Ber is farther from the carboxylate group of Glu-253 (4.9 Å) and is 4.5 Å from the hydroxyl group on Tyr-187, potentially contributing to the lower binding affinity of wtBmrR for Ber (Fig. 2D, Table 1). Interestingly, the backbone carbonyl oxygen of P144 is 3.7 Å from the positive charge on Ber, thereby also contributing to charge neutralization. Another notable difference between R6G and Ber binding is the presence of a hydrogen bond between the amide group of the N149 side chain and an ether oxygen on the berberine molecule (Fig. 2C). This hydrogen bond and the dipole-charge interaction with the backbone of P144 contribute to the more favorable enthalpy of the complex and may mitigate the effects of losing the negative charge at residue Glu-253 in the E253A and E253Q mutants (Table 1). Another consequence of the greater distance between the Ber positive charge and Glu-253 negative charge in comparison to the corresponding distance from R6G to Glu-253 is the smaller effect of mutation of Glu-253 negative charge in comparison to the corresponding distances of Glu-253 and Asp-47, and little favorable binding energy would be expected at this distance (20). Indeed, drug binding experiments demonstrate virtually no change in TTP⁺ affinity for either E253A or E253Q (Table 1).

These data demonstrate that in sharp contrast to the BRC domain, the loss of the electrostatic interaction between residue 253 of full-length BmrR and drug results in unpredictable changes in binding affinity, ranging from essentially no change for Ber and TTP⁺ to a significant reduction for R6G. Even within the R6G data there is little predictability as the isosteric, hydrogen bonding capable E253Q mutant binds ~4.5-fold worse than the small, nonpolar E253A protein (Table 1). Clearly, van der Waals and stacking interactions between the

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### TABLE 2

Selected crystallographic data and refinement statistics

|                     | E253Q-DNA | E253A-DNA | R275E-R6G-DNA | R275E-Ber-DNA |
|---------------------|-----------|-----------|---------------|---------------|
| **Data collection** |           |           |               |               |
| Wavelength (Å)      | 1.003     | 0.984     | 0.979         | 0.979         |
| Resolution (Å)      | 47.2-2.8  | 60.4-2.8  | 52.4-2.6      | 86.1-2.7      |
| Observed reflections (#) | 455,058  | 455,932  | 317,101       | 219,227       |
| Unique reflections (#) | 20,900   | 20,870    | 26,429        | 25,132        |
| Completeness (%) (last shell) | 99.3 (99.9) | 98.5 (99.0) | 97.9 (99.6) | 98.9 (98.6) |
| I/|I (last shell) | 10.0 (1.6) | 12.2 (1.7) | 13.2 (1.8) | 7.3 (1.4) |
| Rsym (%) (last shell) | 5.1 (45.4) | 4.5 (46.0) | 4.6 (43.6) | 5.7 (54.4) |

**Refinement**

|                     | E253Q-DNA | E253A-DNA | R275E-R6G-DNA | R275E-Ber-DNA |
|---------------------|-----------|-----------|---------------|---------------|
| Resolution (Å)      | 47.2-2.8  | 60.4-2.8  | 52.4-2.6      | 86.1-2.7      |
| Reflections (no. working set/no. test set) | 19,828/1072 | 19,802/1005 | 25,132/1275 | 22,297/1132 |
| Protein atoms (#)   | 2,288     | 2,276     | 2,289         | 2,289         |
| DNA atoms (#)       | 468       | 467       | 508           | 490           |
| Solvent molecules (#) | 65       | 64       | 100           | 37            |
| Rwork/Rfree (%)     | 22.5/24.8 | 24.6/27.4 | 22.8/26.7     | 22.8/25.8     |
| Average B-factor (Å²) | 65.2       | 70.5       | 61.0           | 83.3           |
| Average B-factor for drug (Å²) | 93.6       | 120.2      |               |               |

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* Rwork = \[\frac{\sum_{j} \left| \langle F_{o} \rangle - \langle F_{c} \rangle \rangle \sum_{j} \left| \langle F_{o} \rangle \rangle \right|}{\sum_{j} \left| \langle F_{o} \rangle \rangle \sum_{j} \left| \langle F_{c} \rangle \rangle \right|}\]

* Rfree = \[\frac{\sum_{j} \left| \langle F_{o} \rangle - \langle F_{c} \rangle \rangle \sum_{j} \left| \langle F_{o} \rangle \rangle \right|}{\sum_{j} \left| \langle F_{o} \rangle \rangle \sum_{j} \left| \langle F_{c} \rangle \rangle \right|}\]

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**FIGURE 3.** 2Fo – Fc, simulated annealing composite omit maps of the BmrR-Ber-DNA and BmrR-R6G-DNA complexes contoured at 1σ showing electron density represented as blue mesh for Ber (A) and R6G (B).
BmrR and TPP$^+$ and Ber rather than charge-charge interactions are the major enthalpic contributors to binding affinity (Fig. 2, C and E, Table 1).

Interestingly, these drug-bound structures reveal several key differences between the multidrug binding pocket of BmrR and those of the TetR family members QacR, CmeR, YfIR, and TtgR. The cone-shaped BmrR drug binding pocket has a volume of $\sim$750 Å$^3$ and consists of only a single drug binding site. This contrasts sharply to the larger and multifaceted drug binding pockets of QacR and TtgR, which can accommodate drugs in separate but overlapping sites. Indeed, the volumes of the QacR, CmeR, YfIR, and TtgR binding pockets are 1100 Å$^3$, 1000 Å$^3$, 1200 Å$^3$, and 1500 Å$^3$, respectively, nearly twice the volume of the BmrR pocket (6, 8, 30, 31). In addition, the smaller drug binding pocket of BmrR is quite rigid; there are no significant changes in the positions of side chains within the drug binding pocket upon binding different drugs or even imidazole. This is quite distinct from the buried and highly plastic drug binding pockets of QacR and TtgR and likely CmeR and YfIR.

E253A and E253Q BmrR Mutants Are Constitutively Active—To explore the role and importance of residue 253 in the function of BmrR in vivo, the E253A and E253Q substitutions were incorporated into the chromosomal copy of the bmrR gene, and the effect of these mutations on drug resistance was assayed. Substitution of Glu-253 to either alanine or glutamine resulted in an increase in B. subtilis drug resistance, suggesting an increased expression and production of the Bmr multidrug efflux transporter. The minimal inhibitory concentration of ethidium bromide, a typical Bmr substrate, rose from 4 μg/ml (wild type) to 12 μg/ml (E253Q or E253A). Unlike the wild type BmrR bacteria, the resistance of which is elevated to ethidium in the presence of the inducers R6G and TPP$^+$ (9), the resistance of the E253A and E253Q expressing strains does not respond to these inducers but remained at the same already high level (data not shown).

These in vivo experiments suggest that replacement of Glu-253 with either glutamine or alanine converts BmrR into a constitutive activator of bmr expression. To verify this hypothesis, we compared the ability of purified wtBmrR, E253A, and E253Q to activate transcription from the bmr promoter in vitro. The results of the run-off transcription experiments show that wtBmrR alone is able to activate transcription slightly but inconsistently in the absence of drug (Fig. 1A, lanes 2 and 5). The addition of either R6G or Ber leads to a reproducible 2–3-fold activation of transcription (lanes 3, 4, 6, and 7). A similar result was seen for TPP$^+$ (data not shown). Notably, both the E253A and E253Q mutants display an ~4-fold increase over basal wild type levels in the absence of inducer, and the addition of R6G does not produce an additional stimulatory effect (lanes 8–11). These results demonstrate that substitution of Glu-253 to either glutamine or alanine converts the protein into a constitutive transcriptional activator. Intriguingly, mutation of Glu-253 to aspartate does not convert BmrR into a constitutive activator (lanes 12 and 13).

Crystal Structures of Constitutively Active BmrR Mutants—The crystal structure of apoBRC (drug free) reveals that Tyr-152 resides at the N terminus of helix 06, which follows a loop that covers the drug binding pocket (Fig. 4A) (23). Tyr-152 is engaged in a hydrogen bond to the side chain of Glu-253 and occupies the drug binding pocket in a manner that is analogous but not identi-
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FIGURE 5. Changes in the drug binding pocket of Glu-253-substituted proteins, which result in constitutive activation. A, hydrogen bonding network between Tyr-152, Tyr-187, and Tyr-229, and Glu-253 in the apoBRC structure. apoBRC is shown as green ribbons with selected residues depicted as green sticks. Close contacts and hydrogen bonds are represented by dashed lines. Selected oxygen atoms are colored red. B, superimposition of apoBRC and the E253A-DNA complex. E253A is shown in magenta, and selected residues are depicted as sticks. The dashed line between apoBRC residue Tyr-152 and E253A residue Tyr-229 indicates the steric clash that would occur if the Tyr-152 side chain does not move. The thin solid black lines emanating from the Tyr-152 label point to the different locations of this residue in the apoBRC and E253A structures. C, superimposition of apoBRC and the E253Q-DNA complex. E253Q is shown in blue with selected residues depicted as sticks. The dashed line between apoBRC residue Tyr-152 and E253Q residue Tyr-229 indicates a steric clash that would occur if the Tyr-152 side chain did not relocate. The thin solid black lines emanating from the Tyr-152 label points to the different locations of this residue in the apoBRC and E253Q structures.

As expected, these structures take the activated conformation, the Glu-253-Tyr-152 bond is broken, and both this residue and α6 move ~12 Å from their apoBRC positions, allowing the formation of and access to the multidrug binding cavity (Fig. 4A, inset). To understand how substitution of residue Glu-253 by alanine or glutamine leads to constitutive activation, which inverts disruption of the Tyr-152-Glu-253 interaction, the structures of the drug-free E253A-DNA and E253Q-DNA complexes were determined to 2.8 Å resolution (Table 2).

As expected, these structures take the activated conformation and are nearly identical to all BmrR-drug-DNA structures with r.m.s.d. from 0.39 to 0.58 Å over all Cα atoms. The structures of the DNA and all protein-DNA contacts are also identical in these complexes. Again, the side chains of residues within and surrounding the multidrug binding pocket take nearly identical positions. However, small but functionally relevant movements are seen near residue 253 (Fig. 4B). Clearly, if residue Glu-253 is needed to hold Tyr-152 in the pocket to shut down transcription, the loss of all hydrogen bonding capability of the E253A side chain should result in constitutive activation; the E253A-DNA structure supports this idea whereby the Tyr-152 side chain has moved out of the drug binding pocket to its drug-free location (Fig. 5, A and B). Moreover, the loss of hydrogen bonds between Glu-253 and the Tyr-187 and Tyr-229 side chains results in a 1.5 Å shift of the latter side chain, allowing a Tyr-187→Tyr-229 hydrogen bond. In the apo form, similar repositioning of the Tyr-229 side chain would result in a steric clash with the Tyr-152 hydroxyl (Fig. 5B).

The E253Q-DNA structure reveals a similar relocation of the Tyr-152 side chain (Fig. 5, A and C). This unexpected movement is the result of the slightly larger size of the amide group of the glutamine side chain, which causes the side chain to shift by 0.8 Å with respect to the position of the wtBmrR side chain (Fig. 5C). This movement weakens the Tyr-187-E253Q hydrogen bond, but more important, if the drug-free conformation were taken would position the Tyr-152 hydroxyl group within ~2.9 Å of the phenol ring of Tyr-229, resulting in steric clash, a result again supporting the idea that a key step to transcription activation is the disruption of the Tyr-152-Glu-253 interaction (Fig. 5C).

The Role of Tyr-152 in Transcription Activation—To test whether or not disruption of the Glu-253 and Tyr-152 interaction alone induces constitutive activation by BmrR, Tyr-152 was mutated to phenylalanine, and the ability of the resulting protein (Y152F) to activate transcription in vitro measured. Unexpectedly, Y152F was not constitutively active (Fig. 1A, lanes 14–17) thereby demonstrating that disruption of the hydrogen bond between Tyr-152 and Glu-253 alone is insufficient to expel Tyr-152 from the drug pocket and activate transcription. The apoBRC structure provides a rationale for this result; Tyr-152 not only interacts with Glu-253 but also makes van der Waals and aromatic stacking interactions with residues Leu-155, Tyr-187, Tyr-224, Tyr-229, Tyr-268, and Ile-255 (Fig. 5A). Furthermore, the backbone carbonyl oxygen of Tyr-152 hydrogen bonds to the hydroxyl group of Tyr-170. Combined, these interactions maintain the transcriptionally inactive conformation, and the loss of one interaction is not apparently enough to switch conformations. By contrast, in the drug-bound conformation the Tyr-152→Tyr-170 hydrogen bond is lost as the phenolic side chain of Tyr-170 has rotated out of the drug binding pocket to engage in a new hydrogen bond with the side chain carboxylate of Asp-54′ (Fig. 4A). The Asp-54′ side chain also interacts with the εNH3 group of Lys-156, which resides on α6, the helix that moves along with Tyr-152 upon drug binding and transcription activation. Thus, despite the loss of the “anti-activating” Glu-253-Tyr-152 interaction, the Y152F mutant is not constitutively active in the absence of drug due to the loss of the “activating” Tyr-152–Tyr-170→Asp-54′–Lys-156 network. Clearly expulsion of Tyr-152 and α6 from the drug binding pocket is critical for transcription activation.

Reduced Binding Affinity of R6G by the E253Q Mutant—The structures of the wtBmrR-R6G-DNA and E253Q-DNA com-
Structures of BmrR-Drug Complexes

The drug binding data presented here reveal that Glu-253 is not utilized equally by all drugs. Moreover, discounting unforeseen steric effects, removal of the negative charge from this position has a surprisingly small impact on drug binding affinity. Thus, the need for a specific acidic residue is not an absolute requirement for BmrR to bind all cationic toxins. Indeed, in the QacR-pentamidine complex, one of the positively charged benzamidine moieties is not close to any acidic residue of the multidrug binding pocket, and charge neutralization and binding is effected by dipole-charge rather than charge-charge interactions (24). An attractive hypothesis for maintenance of the observed near-normal drug binding affinities is that the electronegative character of the whole binding pocket of BmrR and possibly other multidrug-binding proteins is important for binding cationic compounds.

Calculation of the electrostatic surface potentials of the drug binding pockets for wtBmrR, E253A, and E253Q highlights the overall electronegative character of the binding pocket even in the absence of a negative charge at residue 253 (Fig. 6). Potential contributors to the negative electrostatic field of the BmrR drug binding pocket are residues Asp-47' from the DNA binding domain of the other subunit and Glu-266 on a nearby loop. When mutated singly to alanine, both Asp-47' A and E266A bind R6G with wild type affinity (data not shown). By contrast, the Asp-47' A/E253A double mutant binds R6G ~18-fold less well than wild type BmrR and more than 3-fold less well than the E253A protein, supporting the idea that the overall electronegative character of the binding pocket significant contributes to binding affinity of cationic lipophilic compounds and provides a built in charge redundancy. This finding is in contrast to the multidrug-binding proteins MdfA, EmrE, TtgR, and AcrR whereby a specific charged residue is key to high affinity drug binding (6, 25, 33, 34). Seemingly paradoxical, the more distal the positive charge of the drug is from BmrR residue Glu-253, the weaker the overall drug binding affinity, i.e. R6G > Ber > TPP+ (Table 1, supplemental Fig. S3). This would suggest that Glu-253 can enhance drug binding affinity in a distance-dependent manner. Examination of the thermodynamics of binding discounts this simple generalization, as R6G and TPP+, the strongest and weakest BmrR binders, display equal enthalpies, whereas Ber shows the largest favorable ΔH (Table 1). A more likely explanation for these binding affinity observations is embedded in the rigidity of the BmrR multidrug binding pocket, which unlike QacR, CmrR, YfIR, and TtgR, is not composed of overlapping mimopockets or conformationally flexible side chains (6, 8, 24, 30, 31). Rather for BmrR, cationic drugs appear to position themselves onto a fixed side chain scaffold to maximize stereochemical and geometric complementarity at the expense of charge-charge interactions. From these data we would suggest that perhaps the more important role for residue Glu-253 and perhaps Asp-47' and Glu-266 is to preclude negatively charged and neutral compounds from binding and activating BmrR.

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