Mechanism of Ligand Recognition by BmrR, the Multidrug-Responding Transcriptional Regulator: Mutational Analysis of the Ligand-Binding Site†

Nora Vázquez-Laslop, Penelope N. Markham,‡ and Alex A. Neyfakh*

Center for Pharmaceutical Biotechnology (M/C 870), University of Illinois at Chicago, 900 South Ashland Avenue, Chicago, Illinois 60607

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ABSTRACT: The *Bacillus subtilis* transcriptional regulator BmrR recognizes dissimilar hydrophobic cations and, in response, activates the expression of a multidrug transporter which expels them out of the cell. The structure of the inducer-binding domain of BmrR, both free and in complex with one of the inducers, tetraphenylphosphonium (TPP), revealed an unusual internal binding site, covered by an amphipathic α-helix. Upon unfolding of this helix, the TPP molecule penetrates into the core of the protein, where it contacts six hydrophobic residues and forms an electrostatic bond with a buried glutamate, E134 [Zheleznova et al. (1999) *Cell 96*, 353−362]. Here, a structure-based mutational analysis was used to understand how BmrR interacts with a wide variety of ligands. We determined the effects of alanine substitutions of each of the seven residues interacting with TPP, and mutations within the amphipathic α-helix, on the binding affinities of six different BmrR inducers. The E134A substitution abolished the binding of all but one inducer. Mutations of the hydrophobic residues contacting the ligand, and of the α-helix, had more moderate effects, often with the affinity for some inducers increasing and others decreasing as a result of the same substitution. These results indicate that each inducer forms a unique set of contacts within the binding site. The flexible geometry of this site and the lack of involvement of hydrogen bonds in ligand binding are the likely reasons for the extremely broad inducer specificity of BmrR. The similarly broad substrate specificity of multidrug transporters can be governed by the same structural principles.

Both prokaryotes and eukaryotes possess multidrug-efflux transporters, membrane proteins which recognize numerous structurally diverse toxins and expel them from cells (*1*−*3*). The ability of these transporters to interact with structurally dissimilar compounds challenges the generally accepted view of a highly selective interaction between a protein and a small molecule, e.g., between an enzyme and its substrate, or between a receptor and its ligand. In attempts to solve the problem of multidrug recognition, an extensive mutational analysis has been applied to several multidrug transporters, thus identifying a number of amino acid residues apparently involved in the interaction with the transporter substrates (*4*−*10*). Such information, however, is hardly interpretable without the knowledge of the protein structure. As of yet, due to the technical difficulty of X-ray or NMR analyses of highly hydrophobic multidrug transporters, no structural information for any of them is available.

Recently, an alternative approach to addressing the problem of multidrug recognition has emerged. The expression of the *Bacillus subtilis* multidrug transporter Bmr, which promotes the efflux of structurally diverse hydrophobic cations (*11*), is regulated by BmrR, a DNA-binding protein which binds many of the Bmr substrates and, in response, activates Bmr expression and thus causes expulsion of toxins from the cell (*12*, *13*). BmrR, a member of the MerR family of transcriptional regulators (*14*), is composed of the N-terminal DNA-binding and the C-terminal inducer-binding domains. The individually expressed inducer-binding domain of BmrR, termed BRC for *B*mr*R C*-terminus, retains the ability to bind multiple drugs (*13*, *15*). Being a small (159 amino acid residues) soluble protein, BRC is amenable to crystallographic analysis. The structures of BRC and of its complex with one of the inducers, tetraphenylphosphonium (TPP), have recently been solved, thus revealing for the first time the ligand-binding site of a multidrug-recognizing protein (*16*).

As determined by crystallographic analysis, the molecule of TPP penetrates into the hydrophobic core of BRC where it forms multiple stacking and van der Waals contacts with the surrounding hydrophobic residues (see Figure 4A). Additionally, the positively charged TPP interacts electrostatically with the unusual, deeply buried glutamate residue, E134. The binding of TPP is accompanied by a dramatic local conformational change in protein structure. A short amphipathic α-helix, α2, which normally covers the entrance to the binding site, becomes disordered, thus allowing the molecule of TPP to take its place and gain access to both the buried glutamate and the internal hydrophobic residues.

Although the structures of the ligand-free BRC and the BRC−TPP complex revealed the atomic interactions in-

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* To whom correspondence should be addressed. Phone: (312)996-7231. Fax: (312)413-9303. E-mail: neyfakh@uic.edu.

‡Present address: Influx Inc., 2201 W. Campbell Park Dr., Suite 116, Chicago, IL 60612.

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volved in the binding of one of the BmrR ligands, more general questions remain to be answered. It remains unclear whether different ligands interact with exactly the same set of amino acid residues within the binding site or, alternatively, each of them has its own mode of binding. The relative contributions of electrostatic interactions with the buried glutamate and the interactions with the hydrophobic residues of the binding pocket also remain unknown. It is also unclear to what extent the α2 helix contributes to the binding affinity, and whether it plays any role in discriminating between the BmrR inducers and multiple other hydrophobic cations that do not bind to the protein. Answering these questions is critical for identifying the essential features of the multi-ligand-binding mechanism of BmrR, which potentially can be shared by other multidrug-recognizing proteins, including multidrug transporters.

Some of these questions can be directly addressed by accumulating structural information on a number of complexes of BRC with its ligands. This work is ongoing, and the preliminary results indicate that at least one of the BmrR inducers (compound #3 in Figure 2) binds BRC in a manner similar to TPP, i.e., through disordering the α2 helix and gaining access to the same binding pocket (Zheleznova and Brennan, personal communication). Here we used a less precise but more comprehensive approach to determining how multiple BmrR inducers interact with BRC, that is, to analyze the effects of mutations in the binding site on the binding affinities of a number of BmrR inducers. The mutations targeted all the amino acid residues of BRC known to contact the bound TPP molecule and the residues of the α2 helix that appear to be critical for maintaining its secondary structure. This analysis allowed us to evaluate the relative contribution of different protein residues to the binding of different ligands and make preliminary conclusions on the general molecular principles governing the process of multidrug recognition.

EXPERIMENTAL PROCEDURES

Expression and Purification of the Mutant Variants of the BmrR C-Terminal Domain. Single amino acid mutant variants of BRC were generated by crossover PCR. The vector pHPTBRC (*15*), directing the expression of a histidine-patch thioredoxin fused at its C-terminus with the wild-type BRC, was used as a template. Two overlapping parts of the BRC-encoding region were amplified separately, with each part defined by a standard primer, complementary to either upstream or downstream vector sequences nearby the cloning site, and one of the two complementary mutagenic primers. The obtained PCR products were purified from an agarose gel, and their mixture was used as a template for the third PCR reaction, using the pair of the standard primers. The resulting DNA fragment was then cloned between the *Kpn*I and *Pst*I sites of the pHPTrxFus expression vector (Invitrogen), yielding a plasmid similar to pHPTBRC but carrying the desired mutation within the BRC-coding region. All PCR reactions were performed with the high-fidelity BioExact polymerase mixture (Bioline). Presence of the desired mutation and absence of PCR-generated random mutations were checked by sequencing the cloned fragment with the fMol DNA Cycle Sequencing System (Promega).

Fusions of BRC variants with histidine-patch thioredoxin (HPTBRC) were expressed in *E. coli* GI698, by using tryptophan as an inducer, usually at 30 °C (but see below). Purification of the proteins was performed essentially as described for wild-type BRC (*15*). Briefly, cells were suspended in 0.3 M NaCl in 50 mM sodium phosphate buffer, pH 8.0 (binding buffer), and lysed in a French pressure cell (Aminco). Clarified cell lysates were applied to a nickel nitrilotriacetate agarose (Ni-NTA, Qiagen) column and washed with the binding buffer and then with the elution buffer (0.3M NaCl, 10% glycerol, 50 mM sodium phosphate, pH 6.0). HPTBRCs were eluted with a 0−0.2 M imidazole gradient in the elution buffer and dialyzed against 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol. BRC proteins were separated from the fusion partner by digestion with trypsin. The protease was removed by passing the digest mixture through an agarose-bound trypsin inhibitor (Sigma) column, and pure BRC was obtained by absorbing histidine-patch thioredoxin on a Ni-NTA column. The induction of three of the BRC-mutant variants, L36A, Y68A, and I71A, needed to be performed at 15 °C to avoid intracellular aggregation of the protein. In addition, they became sensitive to trypsin digestion, so enterokinase (EKmax, Invitrogen) was used to separate thioredoxin from these BRC mutants. Purified BRC variants were stored at −20 °C until used.

Tyrosine Fluorescence Measurements of BRC. Fluorescence measurements of wild-type BRC and its mutants were performed in a Shimadzu RF-1501 spectrofluorometer at λ_{ex} 280 nm and λ_{em} 305 nm in 2 mL of 20 mM Tris, pH 7.5, 100 mM NaCl. Protein was added at a concentration of approximately 5 μg/mL to obtain 400 fluorescence units. Additions of 1−2 μL of stock solutions of compounds in DMSO were used for titration of the BRC fluorescence quenching. Compounds used are shown in Figure 2. Rhodamine 6G (compound #1) was from Sigma; 4-amino-3,6-dimethylbenzo[*b*]cycloheptano[*e*]pyridinium chloride (compound #2), 5-(1-adamanthylcarboxyethyl)-3-benzyl-4-methylthiazolium chloride (compound #3), and 5,6-dichloro-1,3-diethyl-2-(phenylaminovinyl)benzoimidazolium chloride (compound #4) were obtained from ChemBridge (Palo Alto, CA); Astrazon Orange (compound #5) and 1,1′-diethyl-2,4′-cyanine iodide (compound #6) were from Aldrich.

For K_D determination, the relative fluorescence quenching, ΔF^*, was adjusted for the quenching effects of compounds on the fluorescence of a solution of free tyrosine (1.5 μM) dissolved in the same buffer. The following formula was used:

$$\Delta F^* = [F_{Po} - F_{Pc}(F_{Yo}/F_{Yc})]/F_{Po}$$

where F_{Po} = fluorescence intensity of a BRC variant, F_{Pc} = fluorescence intensity of BRC in the presence of a particular concentration of the compound, F_{Yo} = fluorescence of tyrosine solution with no compound present, and F_{Yc} = fluorescence of tyrosine solution in the presence of the corresponding concentration of the compound. It should be noted that this formula allows for accurate ΔF^* determination even if the compound itself exhibits moderate fluorescence at the wavelengths used. The obtained values of ΔF^* were plotted against the compound concentration in double-reciprocal (Lineweaver−Burk) plots.

In Vitro Transcription Experiments. Purification of the *B. subtilis* RNA polymerase and in vitro runoff transcription were performed essentially as described previously (*17*), with

the exception that heparin was omitted from the reaction. A 221 bp long PCR product containing the *bmr* promoter in its middle was used as a template and was obtained by amplifying a fragment of the *B. subtilis* genomic DNA. BmrR carrying His-tag at its C-terminus was purified on a Ni-NTA resin from the lysate of the *E. coli* LMG194/pBmrR cells induced with arabinose. The arabinose-inducible pBmrR expression vector was obtained by cloning the BmrR-encoding DNA region between the *Nco*I and *Sal*I sites of the vector pBAD-MycHis (Invitrogen). Transcription reactions (50 μL) contained 90 pmol of BmrR and 2.5 nmol of compounds #1−6. The runoff transcripts labeled with [α-^{32}P]-UTP were separated in a 6% polyacrylamide gel containing 7 M urea and visualized by autoradiography. Ribosomal 5S RNA from *Thermus aquaticus* (125 nucleotides long; kindly provided by Dr. P. Khaitovich) and valine-tRNA from *E. coli* (76 nucleotides long, Sigma) were used to verify the size of the 98 nucleotide long transcription product.

RESULTS

Use of Tyrosine Fluorescence Quenching for Assessing Binding Affinities of BRC Ligands. The goal of the present work was to determine the effects of mutations within the ligand-binding site of BRC on the binding affinities of multiple BRC ligands. Previously, by using the equilibrium dialysis method, we were able to measure the binding affinity of only one BRC ligand, rhodamine 6G, which is highly fluorescent and therefore easily quantifiable; the affinities of other ligands were estimated only by their ability to compete with rhodamine for binding (*12*, *13*, *15*, *16*). To determine the binding affinities of multiple BRC ligands directly, here we developed a new assay based on the ligand-induced quenching of the tyrosine fluorescence of BRC.

BRC lacks highly fluorescent tryptophan residues but contains 11 tyrosine residues that make the protein fluoresce with spectral characteristics typical of tyrosine (λ_{ex} 280 nm, λ_{em} 305 nm). In initial experiments, we found that the addition of submicromolar to micromolar concentrations of rhodamine 6G quenches the tyrosine fluorescence of BRC in a concentration-dependent saturable manner (Figure 1A). The quenching effect of the same concentrations of rhodamine on the fluorescence of free tyrosine was much weaker. Furthermore, rhodamine exerted a similarly weak quenching effect on the fluorescence of the BRC mutants lacking the buried glutamate E134: the E134Q (not shown) or E134A BRC mutants (Figure 1A). These mutant proteins have been previously shown to lack the ability to bind rhodamine in equilibrium dialysis assays (*16*). These results indicate that the strong quenching of the fluorescence of wild-type BRC by rhodamine is a consequence of its binding to the protein.

Although the precise physical nature of the quenching effect remains unknown, a similar phenomenon has been reported for some other ligand-binding proteins where it was used for binding measurements (*18*, *19*). Indeed, as shown in Figure 1B, a double-reciprocal plot calculated from the fluorescence quenching data allows for an accurate determination of the dissociation constant (K_D) of the rhodamine−BRC complex. The obtained value, 0.8 μM, is close to the K_D value determined previously by the equilibrium dialysis method, 1.9 μM (*15*). In addition to being simple, the fluorescence quenching method yields highly reproducible

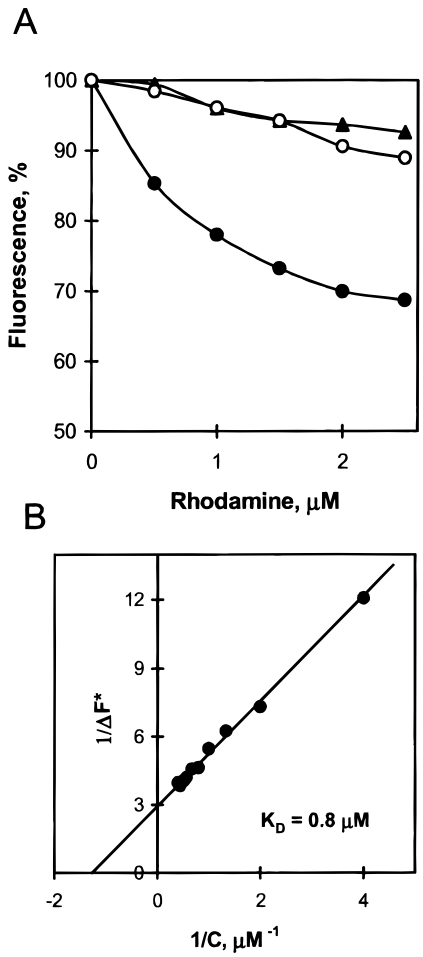

FIGURE 1: Ligand binding by BRC as determined by tyrosine fluorescence quenching. (A) Fluorescence quenching of BRC upon the addition of rhodamine 6G. Fluorescence of the wild-type BRC (closed circles), BRC mutant variant E134A (open circles), or free tyrosine (triangles) was monitored at λ_{Ex} 280 nm and λ_{Em} 305 nm, following the addition of increasing amounts of rhodamine 6G. (B) Determination of the dissociation constant (K_D) of a ligand. The data points from the BRC fluoresecence titration with rhodamine 6G shown in (A) were used to calculate the relative fluorescence quenching (ΔF^*) values with the formula described under Experimental Procedures. These values were fitted in a double-reciprocal plot against the rhodamine concentration in order to determine the K_{Drhod} from the intercept with the abscissa axis. Similar fluorescence titration plots and K_D determinations were generated to evaluate the binding of the hydrophobic cations shown in Figure 2 by wild-type BRC and the mutant variants described in the text.

results. Each K_D determination presented in this work was repeated 2−3 times with the obtained values differing by no more than 20%, even with independently obtained preparations of BRC.

Importantly, the ligand-induced quenching of tyrosine fluorescence was observed not only for rhodamine, but also for all known BmrR inducers and BRC ligands. The only compounds to which this method could not be applied reliably are those which, at the concentrations close to their K_D, significantly fluoresce at 305 nm (which, unfortunately, include TPP) or significantly quench the fluorescence of free tyrosine. Four of the previously identified BmrR inducers and BRC ligands (compounds #1−4 in Figure 2; see refs *12*, *13*) were free from these shortcomings and were used in this work. Additionally, by screening a number of compounds for the ability to quench the tyrosine fluorescence of BRC,

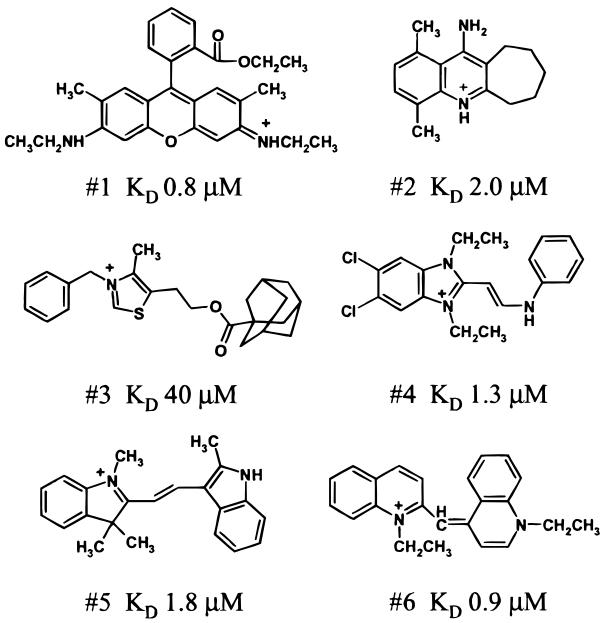

FIGURE 2: Chemical structures of the BRC ligands. Compound #1 (rhodamine 6G) was identified as BRC ligand in ref *15*; compounds #2, 3, and 4 in ref *13*; and compounds #5 and 6 in this study. The K_D values of each ligand for the wild-type BRC are indicated.

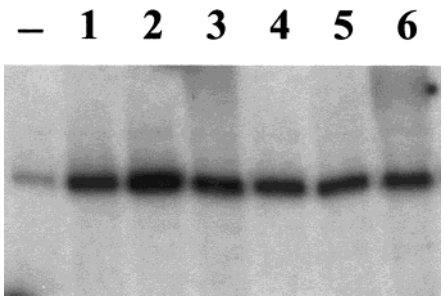

FIGURE 3: Induction of the in vitro runoff transcription from the *bmr* promoter by the BRC ligands (shown in Figure 2) in the presence of BmrR.

we identified two new BRC ligands: dyes Astrazon Orange and diethyl-2,4′-cyanine (compounds #5 and #6 in Figure 2).

All six compounds shown in Figure 2 are BmrR inducers as demonstrated in an in vitro transcription assay (Figure 3). By using the fluorescence quenching method, we determined their binding affinities for the wild-type BRC and 10 of its mutants.

Effects of Mutations in the Ligand-Binding Pocket on Ligand Affinities. In this group of experiments, we constructed seven mutant variants of BRC, each carrying alanine instead of one of the residues known to interact with TPP in the ligand-binding pocket of BRC. As shown in Figure 4A, these residues were the buried glutamate, E134, located at the bottom of the pocket, and hydrophobic residues forming the walls of the pocket: I23, V28, Y51, Y68, I71, and I136.

The wild-type BRC and its mutant variants were expressed as C-terminal fusions with His-Patch thioredoxin, purified by Ni-chelate chromatography, cleaved of the thioredoxin by trypsin, and purified again. Most mutant variants demonstrated the same solubility properties as the wild-type BRC and were insensitive to trypsin digestion used to liberate BRC from its fusion partner. In contrast, mutants Y68A and I71A had to be expressed at 15 °C to avoid their precipitation in the cytoplasm of the *E. coli* cells. Furthermore, these mutant proteins were sensitive to trypsin, and, therefore, their separation from thioredoxin was achieved by digestion with a much more specific protease, enterokinase, which cuts the fusion at the linker region. The K_D values of each of the six tested BRC ligands (Figure 2) for each of the seven mutants and the wild-type BRC were then determined. The obtained results are expressed in Figure 4B as relative affinities, as compared to the wild-type BRC, according to the formula: $K_{Dmut}^{-1}/K_{Dwt}^{-1}$. If this value is lower than 1, this means that the compound binds to the mutant protein with lower affinity (has higher K_D) than to the wild-type BRC. If it is higher than 1, it means that the binding affinity increases as a result of the mutation.

The most dramatic effect was observed for the E134A mutant of BRC, whose binding of almost all compounds tested was practically undetectable. This is consistent with the role suggested for residue E134 as a key electrostatic attractor of hydrophobic cations (16, see the introduction). Remarkably, however, the affinity of compound #3 for BRC was reduced by the E134A mutation only by less than 2-fold, suggesting that the electrostatic interaction established with the buried glutamate 134 is not necessarily critical for all ligands.

Mutations I23A, V28A, Y51A, and I136A showed relatively mild, up to severalfold, effects on the affinities of the ligands. This is consistent with the notion that these residues are engaged in only low-energy hydrophobic contacts with TPP. The most remarkable feature of this set of data is that a single mutation can have disparate effects on the binding of different ligands. An extreme example is the I136A mutation, which reduces the affinity of compound #5 by more than 5-fold, but increases the affinity of compound #3 by more than 3-fold.

Mutations Y68A and I71A exerted negative effects on the affinities of all the ligands tested. Since, as described above, these two mutant variants of BRC exhibited signs of partial misfolding (precipitation at high temperatures during the expression in *E. coli*; increased sensitivity to trypsin digestion), these mutations are likely to affect the architecture of the entire binding pocket. Interestingly, however, different ligands lost affinity to these mutants to different extents. For example, the I71A mutation reduced the binding affinity of compound #5 by more than 10-fold, but had less than 2-fold negative effect on the binding of compound #6.

These results, taken together, strongly suggest that different ligands form different contacts with the residues of the binding pocket.

Role of the α2 Helix in Ligand Binding. As described in the introduction, a short helix α2, which is composed of only nine residues and normally covers the binding pocket, undergoes the most profound structural change as BRC binds a ligand: it becomes disordered allowing for the access of the ligand molecule to the binding site. It is conceivable, therefore, that the inducer specificity of BmrR is determined by the structure of this helix: it may unfold in response to only a certain group of ligands. Furthermore, it can potentially contribute significantly to the binding of some of the ligands. Indeed, although completely disordered in the case of TPP (*16*), which binds BRC with a relatively low affinity ($K_D \sim 100$ μM) (*12, 15*), the residues of the unwound α2 helix may potentially form specific contacts with the better

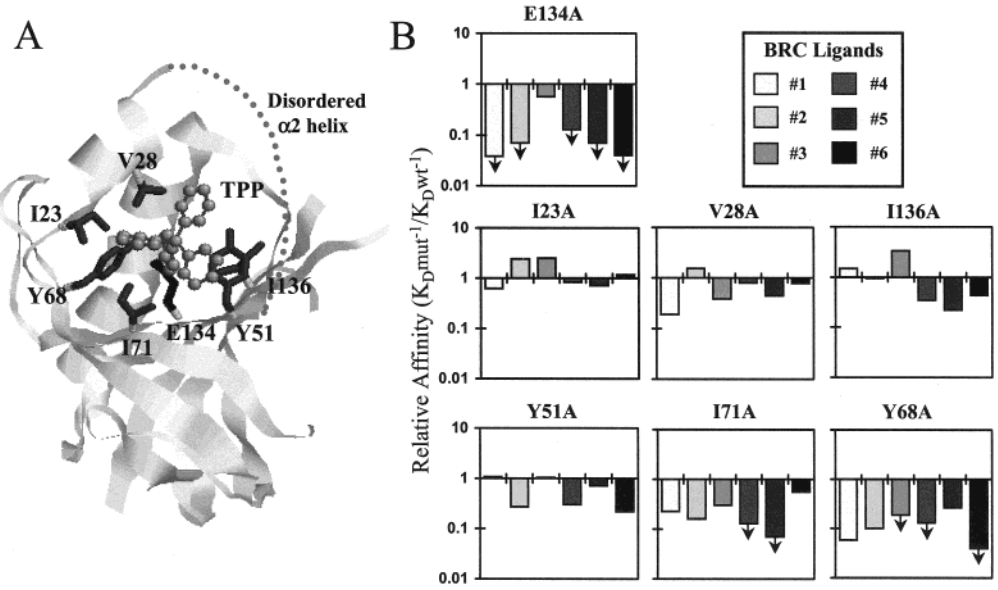

FIGURE 4: Single amino acid mutations in the ligand-binding pocket of BRC and their effects on the affinities for ligands. (A) Positions of the residues in the BRC−TPP complex (*16*) which were substituted by alanine residues. (B) Binding affinities of the BRC mutants, relative to the affinities of the wild-type protein, for the compounds shown in Figure 2. K_D values, calculated as described in Figure 1 and under Experimental Procedures, were used to estimate the relative affinities expressed as $K_{Dmut}^{-1}/K_{Dwt}^{-1}$. Note that these values are expressed on a logarithmic scale. An arrowhead demonstrates that the actual relative affinity can be lower or even much lower than the bar indicates: the binding affinity of a ligand for the mutant BRC was reduced so much that it could not be measured reliably. Bars in these cases were graphed assuming K_{Dmut} to be at the highest detectable level.

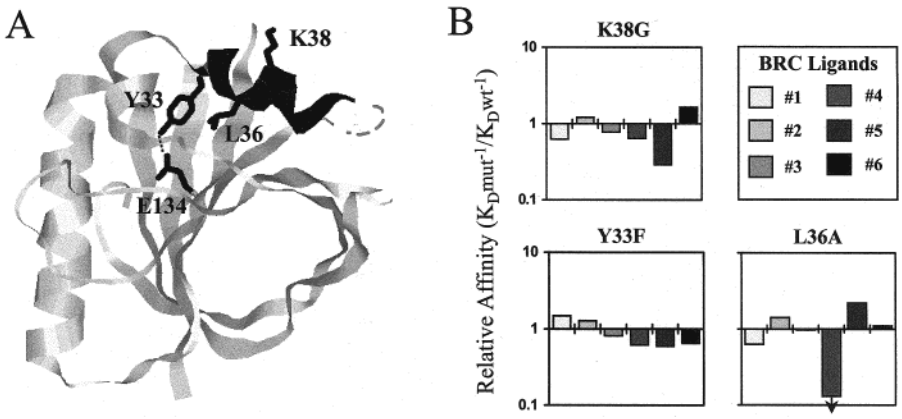

FIGURE 5: Single amino acid substitutions in the α2 helix residues of BRC and their effects on ligand binding. (A) Positions of the three mutated α2 residues in the structure of a ligand-free BRC (*16*): Y33 (mutated to F), L36 (mutated to A), and K38 (mutated to G). (B) Relative affinities of the α2 helix BRC mutants for the compounds shown in Figure 2. See the legend to Figure 4 for details.

BRC ligands. To test these hypotheses, we analyzed three mutant variants of BRC with substitutions in the α2 residues (see Figure 4).

The first mutation, K38G, was chosen because it is likely to disrupt the α-helical folding of the α2 residues. Indeed, glycine is well-known to disrupt α-helices (*20*). Furthermore, the K38 residue is likely to form a salt bridge with another α2 residue, E41, which is located in the next turn of the helix. Since such bridges are known to stabilize α-helical conformation (*21*), its disappearance in the K38G mutant should promote the unwinding of the helix even further. Although verification of the disruptive effect of the K38G substitution on the secondary structure of the α2 residues should await crystallization and determination of the structure of the mutant protein, it is already clear, as illustrated in Figure 5, that this mutation has only moderate effects on the binding affinities of BRC ligands. These effects were significantly different for different ligands: in the most extreme cases, the binding affinity of compound #5 was reduced 3-fold, while the affinity of compound #6 was increased 1.5-fold.

Even smaller effects were detected with another mutant variant of BRC, Y33F. In the ligand-free BRC, the hydroxyl group of tyrosine 33 appears to anchor the α2 helix to the protein by forming a hydrogen bond with the buried glutamate, E134. Upon ligand binding, Y33 undergoes relocation by as much as 11.5 Å and is ejected from the binding pocket (*16*). In the Y33F mutant, the hydrogen bond linking this residue to the buried glutamate should disappear. Yet, the effects of this mutation on the binding affinities of the BRC ligands were barely significant, not exceeding 1.5-fold.

The third mutation of the α2 helix which we explored was the substitution of alanine for residue L36 which, in a ligand-free BRC, penetrates directly into the binding pocket, thus additionally anchoring the α2 helix. This mutant variant of BRC demonstrated high sensitivity to trypsin digestion and had to be separated from thioredoxin in the course of protein purification by using enterokinase. This does not mean,

however, that the mutant protein had defective global folding. Indeed, the L36A mutation had only very small effects on the binding of compounds #1, 2, 3, and 6, and even increased the affinity of compound #5 by a factor of 2. Most dramatically, however, it reduced the binding of compound #4 to an undetectable level, which corresponds to at least 9-fold drop in affinity. It is conceivable that L36 of the unwound α2 helix forms direct contacts with bound compound #4.

In conclusion, the α2 helix appears to play only a limited role in determining the inducer specificity: the binding of only one out of six tested BRC ligands was dramatically affected by a mutation in the helix. Like substitutions within the ligand-binding pocket (see above), the effects of the mutations in the α2 helix were distinct for different ligands, strongly suggesting that each ligand forms a unique set of contacts with the BRC residues.

DISCUSSION

The major result of the mutational analysis of BRC presented in this paper is that each of the tested mutations exerted disparate effects on the binding of different ligands, affecting their binding affinities to different extents, and, in many cases, even in different directions. The only plausible explanation of these results is that different ligand molecules establish different sets of interactions with the residues of the binding site.

The important question here is what allows the binding site of BRC, and maybe of other multidrug-recognizing proteins, to interact with ligands in a number of different modes, whereas a binding site of a regular receptor or enzyme establishes only a specific set of interactions with a small molecule and, therefore, is much more selective. Structural studies of BRC (*16*) and the mutational studies presented here suggest two major reasons for the extraordinary ligand promiscuity of the BRC binding site: the physical nature of the ligand−protein interactions, which are limited to electrostatic attraction and hydrophobic contacts; and the flexible geometry of the binding site.

The results presented here confirmed the critical role of the buried electronegative glutamate residue, E134, in the binding of the majority of the ligands. This was expected from the previously published structure of the BRC−TPP complex and the results showing dramatic negative effects of substitutions of E134 on the binding of rhodamine, as assessed by the equilibrium dialysis method (*16*). A somewhat unexpected finding of the present study is that the binding of one tested BRC ligand, compound #3 (Figure 2), is affected by the E134A substitution only to a small extent, thus suggesting that the electrostatic attraction plays little role in its interaction with BRC. Indeed, the recently determined structure of the BRC−compound #3 complex (Zheleznova and Brennan, personal communication) showed the positive charge of the bound compound #3 to be located as far as 8 Å from the carboxy group of E134. The weakness of the electrostatic bond in this case may be the major reason the affinity of compound #3 for BRC is significantly lower than that of the other ligands tested in this work (K_D of 40 μM as compared to 0.8−2 μM for the other ligands). Yet, this finding shows once again the diversity of the binding modes of different BRC ligands and indicates that the electrostatic attraction is not the only factor in ligand recognition.

The other binding force appears to be multiple stacking and van der Waals contacts which a bound ligand establishes with the hydrophobic residues of the binding pocket. Mutations of these residues produced relatively moderate effects on the ligand-binding affinities, although these effects were again different for different ligands. The only exceptions were mutations Y68A and I71A, which apparently affected the folding of the protein and significantly reduced the binding affinities of many, yet again not all, of the ligands.

Importantly, the physical forces providing for ligand binding to BRC do not include hydrogen bonds. None of the residues of the binding pocket, except for Y51 with its free hydroxyl, has a free chemical group capable of establishing such a bond, and, as we show here, the mutation Y51A has only limited effect on ligand binding. Hydrogen bonds, however, are considered to be the major contributor to the specificity of molecular recognition (*22*), since they have to be of specific lengths and orientations and, therefore, impose severe restrictions on the chemistry of a molecule interacting with a protein. In contrast, the electrostatic attraction, which plays a crucial role in the binding of most ligands to BRC, depends only on the distance between the two charges but not on their orientation and must be especially strong in the hydrophobic environment of the BRC ligand-binding pocket with its very low dielectric constant.

The stacking and van der Waals contacts depend on mutual orientation of interacting molecules and generally require shape complementarity. Importantly, however, the flexible geometry of the binding site of BRC should allow ligands of many different structures to acquire a position within the site with the maximum shape complementarity to the hydrophobic residues of the protein. Indeed, one of the walls of this site is a flexible unfolded α2 helix: ligands of different sizes and shapes can, therefore, fit into the binding pocket. Our results show that, contrary to our expectations, neither the precise nature of the residues composing the α2 helix nor their α-helical propensity has a significant influence on the binding affinities of the majority of the ligands, the only exception being compound #4 whose binding is severely affected by the L36A mutation. Apparently, the role of this amphipathic helix is generally limited to covering the hydrophobic binding pocket from the surrounding water in the ligand-free BmrR, yet being able to easily unfold and allow a ligand to bind. The unfolding of the α2 helix may also serve as an inducing signal to the DNA-binding domain of the full-length BmrR, a possibility which is presently under investigation.

Summarizing, it appears that any small molecule carrying a positive charge, which would be electrostatically attracted to the buried glutamate, and a hydrophobic surface, such that it can find a position within the site to form a large number of contacts with hydrophobic residues, can be a good BRC ligand. This model easily explains why only some hydrophobic cations bind to BRC: the shapes of other molecules may be unsuitable to fit to any of the many allowable positions within the site.

It is tempting to speculate that the recognition of diverse substrates by multidrug transporters follows the same simple rules (see also ref *23*). Indeed, the effects of the mutations described here are very similar phenomenologically to those

reported for mutations in multidrug transporters (*4*−*10*). Like in BRC, many mutations in the transporters affect the transport affinities of different substrates disparately, frequently in different directions. Furthermore, similar to the effects of the E134A substitution, it was demonstrated that mutations of electronegative intramembrane residues of multidrug transporters severely affect the transport of a majority, yet not all substrates (*10*). Since these similarities can be superficial, further biochemical, mutational, and, most importantly, structural studies of multidrug transporters are required to verify these ideas.

An important lesson which the analysis of BRC already provided for the understanding of multidrug transporters is that, contrary to what is frequently assumed, the recognition of multiple compounds by a single protein does not require special or highly unusual molecular interactions. The commonly expressed fascination with the phenomenon of multidrug efflux is, therefore, not necessarily a reflection of its true complexity but rather a consequence of our much greater familiarity with the sophisticated mechanisms of specific molecular recognition, which are characteristic of the majority of proteins studied to date.

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