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The Drug-Binding Activity of the Multidrug-Responding Transcriptional Regulator BmrR Resides in Its C-Terminal Domain

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Rhodamine and tetraphenylphosphonium, the substrates of the *Bacillus subtilis* multidrug efflux transporter Bmr, induce the expression of Bmr through direct interaction with its transcriptional activator BmrR. Here we show that the C-terminal domain of BmrR, expressed individually, binds both these compounds and therefore can be used as a model for molecular analysis of the phenomenon of multidrug recognition.

In recent years, a number of so-called multidrug transporters have been discovered in bacteria (7, 10). Like the eukaryotic multidrug transporters described earlier (3), each of these membrane proteins can mediate active efflux of structurally dissimilar drug molecules from the cell. It remains unknown, however, if the efflux of diverse toxins is the primary function of multidrug transporters or whether they have more specific, but presently unknown, physiological functions.

The normal functions of multidrug transporters can potentially be determined by analyzing regulatory mechanisms controlling expression of these proteins. We have recently shown that the expression of the *Bacillus subtilis* multidrug transporter Bmr (9) is regulated by the transcriptional activator BmrR, which is encoded in the immediate vicinity of the *bmr* gene and binds specifically to the *bmr* promoter (1). BmrR belongs to the MerR family of transcriptional activators. Members of this family, including BmrR, have homologous N-terminal DNA-binding domains. Their C-terminal domains have no homology and are known to be involved in the binding of specific inducer molecules, such as mercury ions for MerR (11) or thiostrepton for TipAL (5).

The natural inducer of Bmr expression which binds to BmrR is unknown. We have found, however, that some of the substrates of the Bmr transporter, namely, rhodamine 6G and tetraphenylphosphonium (TPP), induce the expression of the transporter through direct interaction with BmrR (1). Disruption of the *bmrR* gene completely abolishes this induction phenomenon (1). Furthermore, in vitro experiments demonstrate that rhodamine and TPP increase the affinity of purified BmrR for the *bmr* promoter. Finally, complexes of BmrR with the *bmr* promoter effectively bind rhodamine, while TPP inhibits this binding, perhaps by competing for the same binding site (1).

Considering that rhodamine and TPP are not only structurally dissimilar molecules but are both artificial compounds which *B. subtilis* never encounters in nature, their functional interaction with BmrR suggests that this protein is a unique transcriptional regulator responding to multiple drugs. It was important, therefore, to determine whether rhodamine and TPP behave as genuine inducers, binding to the C-terminal domain of BmrR. In order to answer this question, we have expressed the C-terminal portion of BmrR as an individual protein.

Expression, purification, and properties of the BmrR C-terminal domain. A computer-based prediction of the secondary structure of BmrR strongly indicated a beta turn to be present approximately in the middle (at residues 116 to 119) of the BmrR polypeptide chain. We hypothesized that the region downstream from this turn may constitute a separate domain. This portion of BmrR was genetically fused to the C terminus of *Escherichia coli* thioredoxin in the ThioFusion expression system (6) (purchased from Invitrogen, San Diego, Calif.).

We used a variant of the expression vector pHPTrxFus, in which the thioredoxin sequence was modified to contain a surface histidine patch, facilitating purification of the fusion protein by metal chelate chromatography (8). The DNA fragment, encoding residues 119 to 245 of BmrR and the transcriptional terminator downstream from the gene, was obtained by PCR with *B. subtilis* genomic DNA as a template. The fragment was then cloned between the *Kpn*I and *Bam*HI sites of the pHPTrxFus vector to form a plasmid pHPTBRC (for histidine patch thioredoxin–bmrR C terminus). The fusion protein, HPTBRC, was expressed in *E. coli* GI698 by using tryptophan as an inducer (3 h, 25°C [6]). After the induction, HPTBRC composed up to 20% of the total protein in the soluble fraction of the bacterial lysate (Fig. 1, lane 1).

To purify HPTBRC, cells were resuspended in 0.3 M NaCl in 50 mM sodium phosphate buffer, pH 8.0 (binding buffer), and lysed in a French pressure cell (Aminco). The cell lysate was clarified by centrifugation and applied to a Ni-nitrilotriacetic acid column (Qiagen Inc., Chatsworth, Calif.). After the column was washed with 50 mM sodium phosphate, pH 6.0, containing 0.3 M NaCl, 10% glycerol, and 0.05% Tween 20, HPTBRC was eluted with a gradient of 0 to 0.2 M imidazole in the same buffer, yielding a protein of approximately 95% purity (Fig. 1, lane 2).

BRC was separated from HPT by treating HPTBRC (1 to 3 mg/ml) with 40 μg of trypsin per ml for 30 min at room temperature in 100 mM NaCl–20 mM Tris-HCl, pH 7.4. Trypsin was removed by passing the digestion mixture through a column with an agarose-bound trypsin inhibitor (catalog no. T-7144; Sigma). Trypsin digestion yielded two fragments with apparent molecular masses of 12 and 15 kDa (Fig. 1, lane 3). Western blot (immunoblot) analysis with thioredoxin-specific antibodies and N-terminal microsequencing demonstrated the 12-kDa fragment to be HPT and the 15-kDa fragment to be BRC, cleaved between the Arg-120 and Arg-121 residues of

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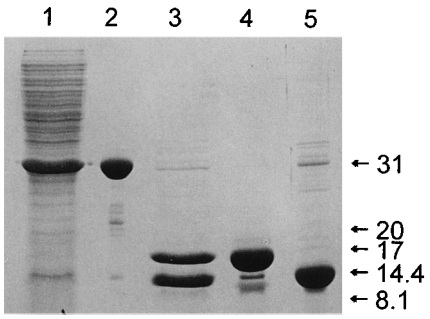

FIG. 1. Purification of HPTBRC and BRC. Shown is a Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel. Lane 1, soluble fraction of *E. coli* expressing HPTBRC; lane 2, HPTBRC purified by nickel chelate chromatography; lane 3, HPTBRC, trypsin digest; lane 4, BRC; lane 5, HPThioredoxin and undigested HPTBRC retained by the nickel chelate column. Numbers at right are molecular weight markers.

the BmrR polypeptide chain. BRC, containing residues 121 to 245 of the BmrR sequence, was subsequently purified to approximately 95% purity by passing the digestion mixture through a Ni-nitrilotriacetic acid column, which absorbed HPT and undigested HPTBRC but not BRC (Fig. 1, lanes 4 and 5).

Since BmrR and its homologs, MerR and TipAL, bind to their target promoters as dimers, it was interesting to determine whether BRC also dimerizes. The results of gel filtration analysis of BRC and HPTBRC were consistent with both existing as dimers. In Sephacryl S200HR chromatography, HPTBRC migrated with an apparent molecular mass of 57 kDa, which is close to the theoretical molecular mass of the dimer (55 kDa). For BRC, the observed and theoretical values were 36 and 28.5 kDa, respectively. These data strongly indicate that the C-terminal domain of BmrR contains a dimerization site, the first result of this kind for any member of the MerR protein family.

Rhodamine 6G and TPP bind the BmrR C-terminal domain. We have previously demonstrated that rhodamine 6G, which can be quantified fluorimetrically, binds BmrR-promoter complexes in equilibrium dialysis ligand-binding experiments (1). The same technique was used to determine whether rhodamine binds to HPTBRC and BRC. As shown in Fig. 2, rhodamine bound these proteins with affinities (K_D, 1.0 to 1.9 μM similar to that previously reported for BmrR-promoter complexes (K_D, 1.2 to 1.8 μM [1]). It should be noted that neither thioredoxin nor other proteins (carbonic anhydrase or total soluble *E. coli* proteins) bind rhodamine in measurable amounts (data not shown; see also reference 1).

Scatchard analysis indicated the number of binding sites for rhodamine to be close to one per BRC or HPTBRC molecule or two per dimer (Fig. 2). This contrasts with the ratio of one rhodamine per dimer previously observed for BmrR-*bmr* promoter complexes (1). A possible explanation for this discrepancy is that binding of BmrR to the *bmr* promoter changes the conformation of its inducer-binding domain so that only one binding site remains accessible to the ligand. Neither of these two ratios, however, is inconsistent with the stoichiometry of inducer binding for other members of the MerR family. For example, MerR binds one mercury ion per dimer (4, 11), while each molecule of TipAL binds one thiostrepton molecule (2a).

TPP, another ligand of BmrR, binds this protein with an affinity lower than that of rhodamine, and its binding is difficult to measure directly in an equilibrium dialysis assay (1). To determine whether TPP interacts with BRC, we analyzed its effect on rhodamine binding. As shown in Fig. 3, increasing concentrations of TPP decreased the affinity of HPTBRC for rhodamine whereas the number of binding sites for rhodamine remained invariant. These data reveal a competitive mechanism of inhibition, first indicating that BRC interacts not only with rhodamine but also with TPP and second suggesting that TPP and rhodamine bind the same or overlapping sites within BRC.

Conclusions and future prospects. Our results demonstrate that the C-terminal domain of BmrR, like the full-length protein, interacts with the inducers of Bmr expression, rhodamine and TPP. This finding strongly indicates that these artificial compounds behave similarly to the natural inducers of the BmrR homologs, MerR and TipAL, which also interact with the C-terminal portions of these proteins.

One possible explanation for the ability of BmrR to respond functionally to artificial compounds is that the Bmr transporter and its regulator, BmrR, have specifically evolved to cleanse the cell of exogenous toxins which are diverse in structure. To fulfill this role, both have acquired a uniquely nonspecific mechanism of molecular recognition. The alternative and biochemically more sound explanation is that rhodamine and TPP merely mimic the presently unknown natural inducer of Bmr expression. This hypothesis is indirectly supported by our recent finding of another *B. subtilis* transcriptional activator, BltR, which regulates expression of Blt, a multidrug transporter homologous to Bmr (2). Although BmrR and BltR both belong to the MerR protein family and have homologous N-terminal domains, BltR, in contrast to BmrR, does not respond to rhodamine or TPP, and it has no sequence similarity to BmrR in its C-terminal domain. This suggests that the normal functions of BmrR and BltR are to recognize different and, probably, specific molecules.

The availability of the drug-recognizing domain of BmrR opens a new avenue for analyzing the enigmatic molecular mechanism of multidrug recognition. The membrane associa-

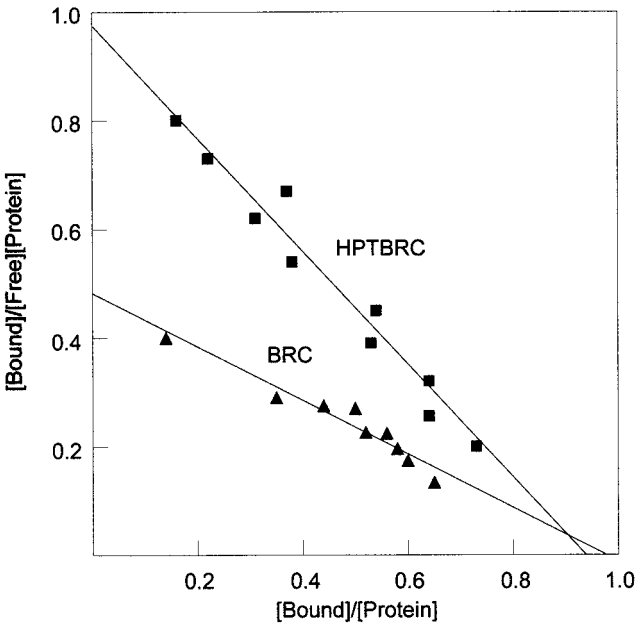

FIG. 2. Scatchard analysis of rhodamine 6G binding to BRC (triangles) and HPTBRC (squares). "[Free]" and "[Bound]" refer to the concentrations of, respectively, free and bound rhodamine. Proteins were used at 5 μM concentrations. As indicated by the points of interception with the abscissa, 1 mol of BRC or HPTBRC binds approximately 1 mol of rhodamine.

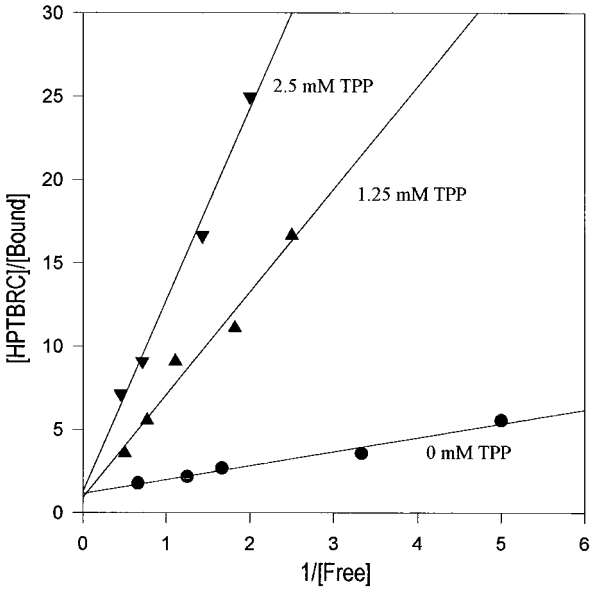

FIG. 3. Rhodamine and TPP compete for binding to HPTBRC. Shown is a double reciprocal plot of rhodamine 6G binding to HPTBRC in the presence of different concentrations of TPP. All the designations and experimental conditions are the same as for Fig. 2.

tion of multidrug transporters and the tendency of BmrR to aggregate unless bound to its target promoter (1) hinder investigation of these proteins. In contrast, the small size (125 amino acid residues) and high level of solubility of BRC make it suitable for applying methods of mutational, biochemical, and structural analyses that can unravel the molecular nature of its multidrug recognition site.

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