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Preliminary structural studies on the multi-ligand-binding domain of the transcription activator, BmrR, from *Bacillus subtilis*

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Abstract

In the bacterium *Bacillus subtilis*, the DNA-binding regulatory protein, BmrR, activates transcription from the multidrug transporter gene, *bmr*, after binding either rhodamine or tetraphenylphosphonium. These two compounds, which have no structural similarity, are also substrates for the bacterial multidrug transporter. BmrR belongs to the MerR family of transcription activators but differs from the other family members in its ability to bind unrelated small molecule activators. As an initial step in the elucidation of the mechanism by which BmrR recognizes rhodamine and tetraphenylphosphonium and activates transcription, we have crystallized the 144-amino acid-residue carboxy terminal dimerization/ligand-binding domain of the BmrR, named the BRC (BmrR C-terminus). Tetragonal crystals of ligand-free BRC take the space group $P4_12_12$, or its enantiomorph $P4_32_12$, with unit cell dimensions $a = b = 76.3$ Å, $c = 96.0$ Å, $\alpha = \beta = \gamma = 90°$. Diffraction is observed to at least 2.7 Å resolution at room temperature. In addition, we determined the secondary structure content of ligand-free and rhodamine-bound BRC by circular dichroism. In the ligand-free form, BRC has considerable β-sheet content (41%) and little α-helix structure (13%). After BRC binds rhodamine, its β-sheet content increases to 47% while the α-helix structure decreases to 11%. The structure of BRC will provide insight not only into its multidrug recognition mechanism but could as well aid in the elucidation of the recognition and efflux mechanisms of Bmr and other bacterial multidrug transporters.

Keywords: BRC; circular dichroism; crystallization; multidrug recognition; transcription activator; X-ray diffraction

Multidrug resistance is observed clinically as the unresponsiveness of organisms, both prokaryotes and eukaryotes, to a variety of chemical therapeutics. One of the major mechanisms by which multidrug resistance arises is the active efflux of drugs and toxins via membrane transporters (Gottesman & Pastan, 1993; Lewis, 1994). Such active transport results in the inability of drugs to affect their intracellular targets and, thus, in the failure of the drug therapy. These transporters, which are called multidrug transporters (mdr) because of their remarkable ability to recognize and pump out structurally and chemically diverse toxic compounds, have gained considerable attention. Although studied extensively in eukaryotes (Gottesman & Pastan, 1993), mdrs are also common to bacteria (Lewis, 1994; Nikaido, 1994; Paulson et al., 1996). Yet, in spite of intensive molecular and biochemical studies, the structural mechanisms by which multidrug transporters recognize their dissimilar substrates remain unclear (Gottesman & Pastan, 1993). Undoubtedly, understanding this mechanism at the atomic level could open novel avenues to overcome drug resistance. However, these membrane-associated mdr transporters have yet to be purified in the amounts necessary for crystallographic studies.

In addition and as an alternative to the bacterial multidrug transporters, their transcription regulators represent potential targets for chemotherapeutic intervention. Furthermore, these cytosolic proteins can serve as readily available systems to elucidate the biochemical and structural mechanisms of multi-ligand recognition. One candidate for these studies is the 279-amino acid-residue protein BmrR from *Bacillus subtilis*. This dimeric protein activates transcription from the bacterial mdr transporter gene, *bmr* (Neyfakh et al., 1991) after binding either rhodamine or tetraphenylphosphonium. These structurally dissimilar compounds are also substrates for the *B. subtilis* mdr transporter, Bmr (Ahmed et al., 1994). Therefore, *B. subtilis* carries a two-fold defense system against multiple toxic compounds in which not only the multidrug transporter but also its regulatory protein are mobilized to effect the removal of structurally diverse compounds (drugs) from the cell.

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The amino terminal region of BmrR contains a putative helix-turn-helix motif and shows substantial sequence homology to the amino terminal DNA-binding domains of a family of bacterial regulatory proteins, which include SoxR from *Escherichia coli* (Gaudu & Weiss, 1996; Hidalgo et al., 1997), the mercury resistance operon regulator, MerR, of several bacterial species (Summers, 1992; Ansari et al., 1995; Utschig et al., 1995) and $TipA_L$ from *Streptomyces lividans* (Holmes et al., 1993). Whereas the carboxy terminal activation domains of the latter two proteins bind a single specific ligand, BmrR is different in its high-affinity binding of the structurally dissimilar molecules, rhodamine (K_d = 1.4 μM) and tetraphenylphosphonium ($K_d \approx 100$ μM) (Ahmed et al., 1994). Recently, four additional cationic aromatic compounds have been identified as potential ligands (Markham et al., 1997). The structural mechanisms by which members of this family bind their cognate promoters and small molecule activators is still poorly understood.

As an initial step in the elucidation of the stereochemical mechanism of multi-ligand binding by BmrR, a 144-amino acid-residue carboxy terminal domain of BmrR (abbreviated as BRC), which is responsible for dimerization and high affinity multi-ligand binding, has been crystallized. Furthermore, to investigate any ligand-induced changes in the secondary structure content of BRC, circular dichroism studies have been done on the ligand-free and rhodamine-bound BRC.

Results and discussion

Tetragonal bipyramidal crystals of BRC typically grow to dimensions of 0.4 mm × 0.4 mm × 0.3 mm and take the space group $P4_12_12$ or its enantiomorph $P4_32_12$. The unit cell dimensions are: $a = b = 76.3$ Å, $c = 96.0$ Å and $\alpha = \beta = \gamma = 90°$. This crystal form diffracts isotropically to at least 2.7 Å at room temperature. Intensity data have been collected on an ADSC multiwire area detector and processed with software provided by ADSC (Table 1).

Crystals of the selenomethionine-substituted BRC diffract to 3.0 Å at room temperature. An intensity data set on these crystals has been collected (Table 1). Difference Patterson analysis reveals one peak in the asymmetric unit and indicates that the selenomethionine-incorporated BRC is a reasonable heavy atom derivative. As only one site was found, it is very likely that there is a monomer in the asymmetric unit. Interestingly, a monomer per asymmetric unit results in a Matthews' coefficient (V_m) of 4.23 Da/Å^3, which is unusually high for proteins that diffract reasonably well (Matthews, 1968). However, the assumption that there are two molecules per asymmetric unit yields a V_m of 2.17 Da/Å^3, which is less congruous with the diffraction quality of this crystal form. Thus, the number of protein molecules per asymmetric unit remains somewhat ambiguous.

Table 1. *Summary of crystallographic data collected for ligand-free BRC and two heavy-atom derivatives*

	Native	SeMet	$KAuCl_4$
Resolution (Å)	2.7	3.0	3.0
Number of measurements	51,160	25,303	24,171
Number of reflections	11,251	10,513	10,151
Completeness (%)	100	97.3	94.0
R_{sym} (%)[a]	4.9	5.5	6.6
R_{iso} (%)[b]	—	11.0	27.3

[a] $R_{sym} = \Sigma|I_o - \langle I\rangle|/I_o$, where I_o is the observed intensity and $\langle I\rangle$ is the average intensity obtained from multiple observations of symmetry related reflections.

[b] $R_{iso} = \Sigma||F_{PH}| - |F_P||/\Sigma|F_P$, where $|F_P|$ is the protein structure factor amplitude and $|F_{PH}|$ is the heavy atom derivative structure factor amplitude.

Attempts to co-crystallize BRC with its ligands, rhodamine or tetraphenylphosphonium, were unsuccessful. However, we were able to introduce ligands into crystals via soaking for several hours in a crystallization solution that contained 2 to 3 molar excess of these compounds. Overnight soaks resulted in crystal cracking.

The unusually high salt concentration (4.3 M NaCl) of the crystallization solution prompted us to determine the oligomerization state of BRC. Using dynamic light scattering, we found that in the presence of 0.1 M NaCl, a 2 mg/mL solution of BRC is monodisperse and has an estimated molecular weight of 45 kDa. Because the molecular weight of the monomer is 16.5 kDa, this result would indicate a trimeric BRC. However, if a dimeric protein has a non-spherical shape (for instance, elongated) its molecular weight might be overestimated. In addition, the existence of BRC as a dimer is consistent with previous analytical gel filtration studies that showed BRC migrates with an apparent molecular weight of 36 kDa, which is slightly greater than its calculated molecular weight of 33 kDa (Markham et al., 1996). Furthermore, gel mobility shift assays and gel filtration chromatography studies on full-length BmrR-cognate operator complexes (Ahmed et al., 1994) also support the dimeric nature of BmrR and, consequently, of BRC. Additional dynamic light scattering experiments revealed that increasing the BRC concentration to 8 mg/mL in the 0.1 M NaCl solution leads to polydispersity and the formation of aggregates with estimated molecular weights from 300 to 1000 kDa. Interestingly, when the salt concentration is raised to 4 M NaCl, which approximates our crystallization condition, the 8 mg/mL solution of BRC becomes monodisperse again. The molecular weight of the average particle has increased to 180 kDa, which can be rationalized as a tetrameric ensemble of BRC dimers.

The results from these dynamic light scattering experiments parallel our crystallization results, in which crystallization trials containing less than 3 mg/mL protein remain clear, while those made with protein solutions of 10 mg/mL form fluffy precipitates in the presence of 0.1–0.5 M NaCl or produce three-dimensional, data quality crystals when the NaCl concentration is raised to 4.3 M. Our observations support previous dynamic light scattering studies, which indicate that non-discretely aggregating protein solutions do not crystallize readily, whereas monodisperse protein samples can yield diffraction quality crystals (Ferré-D'Amaré & Burley, 1994).

We also determined the secondary structure content of ligand-free and rhodamine-bound BRC by circular dichroism to investigate any ligand-induced changes in the secondary structure of the protein. The CD spectrum of the ligand-free BRC is shown in Figure 1. The variable selection method used to deconvolute this spectrum yielded a secondary structure of 13% helix, 41% β-sheet, 10% β-turn and 36% other structural elements (Table 2). The high β-sheet and low α-helix composition was unexpected as secondary structure prediction methods overestimated the percentage of α-helix by as much as two-fold. To test whether binding of rhodamine induces a structural change in BRC, we extended our analysis to determine the CD spectrum of BRC in the presence of rhodamine. The CD spectrum of rhodamine alone was subtracted from the CD

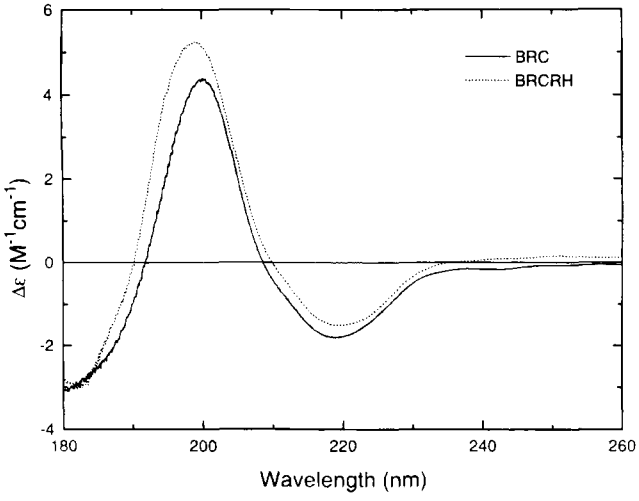

Fig. 1. CD spectra of ligand-free and rhodamine-bound BRC. The change in the molar amino acid ellipticity, $\Delta\epsilon$ (M^{-1} cm^{-1}, ordinate), is plotted against the wavelength (nm, abscissa). The solid line (—) represents the spectrum of ligand-free BRC (BRC) in 50 mM potassium phosphate buffer, pH 7.4; the dashed line (- - -) represents the spectrum of rhodamine-bound BRC (BRC–RH) in the same buffer. The concentration of BRC used for both spectra is approximately 1.3 mg/mL and the concentration of rhodamine in the BRC-RH sample is 0.1 mM. All spectra were recorded at 20 °C.

spectrum of BRC in the presence of rhodamine and the resulting difference CD spectrum of BRC in its ligand-bound form is shown in Figure 1. There is a distinct, though small, change in the CD spectrum of BRC upon binding of rhodamine, which, according to the variable selection method, represents a 6% increase in β-sheet content accompanied by a 2% decrease in the α-helix and a 5% decrease in the β-turn structure (Table 2). We assume that in the presence of BRC the CD signal of rhodamine does not change and, therefore, conclude that the binding of rhodamine to BRC induces or stabilizes β-sheet structure. The crystal structure of the ligand-free and rhodamine-bound BRC will be necessary to confirm this. CD experiments with the BRC ligand, TPP, could not be done because of the strong UV absorption of the ligand at saturating concentration.

Crystallographic studies on BRC will help unravel the stereochemical mechanism by which this protein binds the structurally different compounds, rhodamine and tetraphenylphosphonium. Furthermore, the BRC structure could possibly shed light on the substrate binding site of the multidrug transporter Bmr. The amino acid sequence identity between BRC and Bmr is approximately 26% but the identical, as well as homologous, residues are scattered throughout the sequence. However, this clearly does not preclude the two proteins from having structurally homologous multi-ligand-binding pockets. Such similarity might not be detected by a linear sequence comparison if the ligand-binding sites are formed by the three-dimensional arrangement of non-contiguous amino acid residues. Regardless, the crystal structure of the BRC in its unliganded form and in complex with rhodamine and tetraphenylphosphonium will reveal the atomic architecture of a ligand-binding pocket that favors the binding of multiple, structurally unrelated compounds.

Table 2. *Secondary structure content of ligand-free and rhodamine-bound BRC*[a]

	H	B	T	O	Total
BRC	0.13	0.41	0.11	0.36	1.01
BRC–RH	0.11	0.47	0.06	0.36	1.00

[a]The secondary structure contents determined by the variable selection method for the spectra shown in Fig. 1. The abbreviations used are: H, α-helix; B, β-sheet; T, β-turn; O, other. BRC corresponds to ligand-free protein and BRC–RH is BRC bound to rhodamine.

Materials and methods

Crystallization and intensity data collection

The BRC, which encompasses amino acid residues 121–264 of BmrR, was prepared by trypsin cleavage of a BRC–thioredoxin fusion protein and purified as described previously (Markham et al., 1996). BRC was chosen for the studies as the intact BmrR protein is only sparingly soluble. The pure BRC was concentrated to 10–15 mg/mL in a solution of 20 mM tris·HCl, pH 7.5, and 0.1 M NaCl. Crystals of the apo BRC were grown at room temperature in hanging drops by the vapor diffusion method. The crystallization solution was 4.3 M NaCl, 2 mM $MnCl_2$, and 20 mM tris·HCl, pH 8.5. The presence of Mn^{2+} ions is crucial for the production of diffraction quality crystals (Trakhanov & Quiocho, 1995). Crystals took a month to appear and grew for two additional weeks. Rhodamine or tetraphenylphosphonium was introduced into the crystals by soaking the native BRC crystals for several hours in a mother liquor which contained 1.5 mM rhodamine or 2 mM tetraphenylphosphonium. X-ray intensity data were collected at room temperature with an Area Detector Systems Corporation (ADSC) multiwire area detector using a Rigaku RU200-H rotating anode generator as the X-ray source (40 kV, 150 mA). The data were processed with software provided by ADSC (Xuong et al., 1985) and their relevant statistics are given in Table 1.

Preparation of the heavy atom derivatives

BRC contains two methionine residues and a selenomethionine-substituted BRC was prepared by transforming the BRC–thioredoxin–pBAce construct into *E. coli* DL41 cells (Yale Genetic Stock Center), a strain which is auxotrophic for methionine. The methionine normally included in the low phosphate induction media (Allen & Ullman, 1993) was replaced with selenomethionine at a final concentration of 40 mg/mL (LeMaster & Richards, 1985). Cells were grown in the dark at 30 °C for 24 h. The selenomethionine-substituted protein was purified according to the previously reported protocol (Markham et al., 1996) except for the addition of 5mM DTT to prevent selenomethionine oxidation. The final yield of the selenomethionine-substituted BRC was 3 mg/L. Crystals of the selenomethionine-incorporated BRC grow under conditions described above and are isomorphous with the native crystals. An additional potential heavy metal derivative was prepared by soaking native BRC crystals for 1 h in 5 mM $KAuCl_4$. Relevant crystallographic data of the selenomethionine-substituted and gold-soaked crystals are given in Table 1.

Dynamic light scattering

Dynamic light scattering studies on BRC were done using a DynaPro-801 Dynamic Light Scattering Instrument (Protein So-

lutions). Protein samples at either 2 or 8 mg/mL were prepared in solutions of 20 mM tris·HCl buffer, pH 7.5, which contained either 0.1 M or 4.0 M NaCl. Prior to the experiment, all protein samples were filtered through 0.1 micron Anotop filters (Whatman) to eliminate any aggregated particles. All data were analyzed using AutoPro PC software. The reported values are averages of 10 scans of 30 s each.

CD spectroscopy

CD spectra of BRC in the presence or absence of rhodamine were taken on a JASCO J-500A spectrometer. Measurements were made using a 0.1 mm path length cell (Helma), thermostated at 20 °C using a constant temperature bath (Lauda). The instrument was calibrated with solutions of (+)−10-camphorsulphonic acid ($\Delta\epsilon$ = +2.37 M^{-1} cm^{-1} at 290.5 nm and −4.95 M^{-1} cm^{-1} at 192.5 nm). Data were collected on an IBM/PC-XT using the IF-2 interface and software provided by Jasco. Spectra and buffer baselines were the averages of 10 scans, each recorded at 0.1 nm intervals with a scanning rate of 10 nm/min and a 2-s time constant. The protein concentration was determined by amino acid analysis and was approximately 1.3 mg/mL. The BRC samples subjected to the CD analysis contained either protein in 50 mM potassium phosphate buffer, pH 7.4, or the protein in 50 mM potassium phosphate buffer, pH 7.4., plus 0.1 mM rhodamine. Before spectral deconvolution for secondary structure analysis, the buffer baseline was subtracted and the spectra were smoothed using the smoothing software provided by Jasco. The protein spectrum in the presence of rhodamine is a difference spectrum. The CD spectrum of 0.1 mM rhodamine solution in 50 mM potassium phosphate buffer, pH 7.4, was subtracted from the spectrum of BRC measured in the presence of 0.1 mM rhodamine. To determine the secondary structure content the CD spectra were deconvoluted using the variable selection method described in detail in (Compton et al., 1987).

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References

Allen TE, Ullman B. 1993. Cloning and expression of the hypoxanthine–guanine phosphoribosyltransferase gene from *Trypanosome brucei*. *Nucleic Acids Res 21(23)*:5431–5438.

Ahmed M, Borsch CM, Taylor SS, Vazques-Laslop N, Neyfakh AA. 1994. A protein that activates expression of a multidrug efflux transporter upon binding the transporter substrates. *J Biol Chem 269*:28506–28513.

Ansari AZ, Bradner JE, O'Halloran TV. 1995. DNA-bend modulation in a repressor-to-activator switching mechanism. *Nature 374*:371–375.

Compton LA, Mathews CK, Johnson WC Jr. 1987. The conformation of T4 bacteriophage dihydrofolate reductase from circular dichroism. *J Biol Chem 262*:13039–13043.

Ferré-D'Amaré AR, Burley SK. 1994. Use of dynamic light scattering to assess crystallizability of macromolecules and macromolar assemblies. *Structure 2*:357–359.

Gaudu P, Weiss B. 1996. SoxR, a [2Fe–2S] transcription factor, is active only in its oxidized form. *Proc Natl Acad Sci USA 93*:10094–10098.

Gottesman MM, Pastan I. 1993. Biochemistry of multidrug resistance mediated bythe multidrug transporter. *Annu Rev Biochem 62*:385–427.

Hidalgo E, Ding H, Demple B. 1997. Redox signal transduction via iron–sulfur clusters in the SoxR transcription activator. *TiBS 22*:207–210.

Holmes DJ, Caso JL, Thompson CJ. 1993. Autogenous transcriptional activation of a thiostrepton-induced gene in *Streptomyces lividans*. *EMBO J 12*:3183–3191.

LeMaster DM, Richards FM. 1985. ^{1}H–^{15}N heteronuclear NMR studies of *Escherichia coli* thioredoxin in samples isotopically labelled by residue type. *Biochemistry 24*:7263–7268.

Lewis K. 1994. Multidrug-resistance pumps in bacteria: Variations on a theme. *TiBS 19*:119–123.

Markham PN, Ahmed M, Neyfakh AA. 1996. The drug-binding activity of the multidrug-responding transcriptional regulator BmrR resides in its C-terminal domain. *J Bacteriol 178*:473–475.

Markham PN, LoGuidice J, Neyfakh AA. 1997. Broad ligand specificity of the transcriptional regulator of the *Bacillus subtilis* multidrug transporter BmrR. *Biochem Biophys Res Comm 239*:269–272.

Matthews BW. 1968. Solvent content of protein crystals. *J Mol Biol 33*:491–497.

Neyfakh AA, Bidnenko VE, Chen LB. 1991. Efflux-mediated multidrug resistance in bacteria: Similarities and dissimilarities with the mammalian system. *Proc Natl Acad Sci USA 88*:4781–4785.

Nikaido H. 1994. Prevention of drug access to bacterial targets: Permeability barriers and active efflux. *Science 264*:382–388.

Paulson IT, Brown H, Skurray RA. 1996. Proton-dependent multidrug efflux systems. *Microbiol Rev 60*:575–608.

Summers AO. 1992. Untwist and shout: A heavy metal responsive transcriptional regulator. *J Bacteriol 174*:3097–3101.

Trakhanov S, Quiocho FA. 1995. Influence of divalent cations in protein crystallization. *Protein Sci 4(9)*:1914–1919.

Utschig LM, Bryson JW, O'Halloran TV. 1995. Mercury-199 NMR of the metal receptor site in MerR and its protein-DNA complex. *Science 268*:380–385.

Xuong NH, Nielsen C, Hamlin R, Anderson DJ. 1985. Strategy for data collection from protein crystals using a multiwire counter area detector diffractometer. *J Appl Cryst 18*:342–350.