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Crystal structure of MexZ, a key repressor responsible for antibiotic resistance in *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa is responsible for around 10% of all hospital-acquired infections and the single most important pathogen of cystic fibrosis lungs. *P. aeruginosa* has high intrinsic and acquired antibiotic resistance, due to the extrusion of antibiotics by multidrug efflux pumps. The gene regulator MexZ controls the expression of *mexXY*, the efflux pump responsible for resistance to many drugs that are used for treating CF patients. MexZ is shown to be the most frequently mutated gene in *P. aeruginosa* isolated from CF patient lungs, confirming its importance in multidrug resistance. Here we present the crystal structure of MexZ at 2.9 Å. Combining the structural information with biochemical data on key mutants identified, we provide an explanation for the structural and functional consequences of these mutants. This work provides a framework for further characterisation of MexZ in order to fully understand its regulation and induction.

1. Introduction

Pseudomonas aeruginosa is a clinically challenging, opportunistic pathogen of humans and is responsible for around 10% of all hospital-acquired infections (Fluit et al., 2000; Foundation, 2002; Glupczynski et al., 2001; Govan et al., 2007; Govan and Deretic, 1996; Hancock and Speert, 2000; Van Delden and Iglewski, 1998; Vincent, 2000; Vincent et al., 1995). *P. aeruginosa* has a high association with AIDS and cancer patients (Van Delden and Iglewski, 1998) as well as with neutropenic and mechanically ventilated patients, the latter infections being associated with high fatality rates (Garau and Gomez, 2003). A clinically important characteristic of *P. aeruginosa* is its high intrinsic and acquired antibiotic resistance, due to the low permeability of its outer membrane in combination with the operation of multidrug efflux pumps (Kohler et al., 1999; Poole, 2004; Poole and Srikumar, 2001; Schweizer, 2003). The multidrug efflux pumps are membrane bound transporters that actively expel substrates and contribute significantly to the intrinsic and acquired resistance to antimicrobials in Gram-negative bacteria (Poole, 2004; Poole, 2005; Ramos et al., 2002). MexXY-OprM is a clinically important efflux pump and is involved in resistance to hydrophilic aminoglycosides such as paromomycin, streptomycin, amikacin, gentamicin, and tobramycin and other antibiotics including cefepime and ceftazidime in *P. aeruginosa*, which are commonly used to treat CF patients (Aires et al., 1999; Vogne et al., 2004; Westbrock-Wadman et al., 1999). *mexXY* expression is the predominant cause of aminoglycoside resistance in isolates from CF lungs (MacLeod et al., 2000; Phillips et al., 1986; Poole, 2005; Schumacher et al., 2001; Teran et al., 2006).

MexZ is a negative regulator of *mexXY* expression, with repression being relieved in the presence of inducing antibiotics. The clinical importance of MexZ has been strikingly highlighted by the study of Smith et al. (2006), in which *P. aeruginosa* was isolated from patients during years of chronic infection and the mutational changes that occurred in the clonal population of the bacterium over this period were determined. *mexZ* was found to be the most frequently mutated gene, occurring in *P. aeruginosa* from 18/29 patients studied (Smith et al., 2006), and leading to acquisition of antibiotic resistance. This study highlights the importance of *mexXY* regulation in adaptation to the CF lung environment. An understanding of MexZ's mechanism and regulation could therefore lead to new insights into adaptation and antibiotic resistance.

MexZ belongs to the TetR family of proteins that are characterised by conserved DNA binding sequences, but variable ligand binding domains (Ramos et al., 2005). TetR family proteins exist as dimers and act as repressors by binding to consecutive DNA major grooves through N-terminal HTH DNA binding domains. Upon binding of inducing agents to the C-terminal domain, the DNA binding domains change conformation, hence release the protein from DNA. Many TetR family members, including TetR, QacR, and TtgR, contain a drug binding pocket within the ligand binding domain and bind to the same substrates as the efflux pumps they control (Alguel et al., 2007; Hinrichs et al., 1994; Schumacher et al., 2001; Teran et al., 2006). *mexXY* expression is inducible by

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antibiotics, especially those that target the ribosome (Jeannot et al., 2005). However, these same substrates have no effect on the interaction of MexZ with DNA, suggesting that they do not bind MexZ directly (Matsuo et al., 2004). Instead, an unknown regulator that responds to the antibiotic inducer is proposed to modulate MexZ's activity. Very recently, the gene product PA5471 has been shown to interact with MexZ and the interactions reduced MexZ's binding to DNA, hence increasing the expression of *mexX* (Morita et al., 2006; Yamamoto et al., 2009).

2. Materials and methods

2.1. Protein expression, purification, and crystallisation

Point mutations were introduced using the QuickChange site directed mutagenesis kit (Stratagene). Expression constructs in pET28a encoding *mexZ* WT and point mutations were transformed into *Escherichia coli* BL21(DE3) cells (Novagen). Cells were grown first in 5 ml LB medium overnight and were then inoculated into 500 mL of autoinduction medium (10 g/l tryptone, 5 g/l yeast extract, 20 mM $MgSO_4$, 0.5% glycerol, 0.05% glucose, 0.2% α-lactose, 10 mM $(NH4)_2SO_4$, 20 mM KH_2PO_4, 20 mM Na_2HPO_4) supplemented with 100 μg/ml ampicillin. The cells were initially incubated at 37 °C until OD600 reached 0.6 before reducing temperature to 20 °C for overnight incubation. Cells were harvested by centrifugation and resuspended in lysis buffer (50 mM HEPES, pH 8.0, 500 mM NaCl, 5% (v/v) glycerol, 20 mM imidazole). Cells were lysed by sonication and the suspension was centrifuged at 12,000*g* for 45 min. The protein was then purified using a Ni HiTrap column followed by gel filtration using a Superdex 200 HiLoad. Crystals were grown using the sitting drop vapor diffusion method in 0.2 M K/Na-tartrate, pH 8.5, 300 mM NaCl, 10% glycerol and 20% (v/v) PEG3350 with 10 mg/ml initial protein concentration at 20 °C.

2.2. Crystallographic data collection and structural determination

All crystals were soaked in crystallization buffer supplemented with 20% (v/v) glycerol as cryo-protectant before being frozen in liquid nitrogen. Datasets were collected under cryogenic conditions. Data were processed using XDS (Kabsch, 1993). The crystals are in space group H32 with unit cell dimensions of 177.1, 177.1 and 54.7 Å. Experimental phases were obtained from selenomethionine substituted crystals and Multiple-wavelength Anomalous Diffraction (MAD) methods (Hendrickson et al., 1990). Five selenium sites were located and refined using SOLVE (Terwilliger, 2000). Density modification and phase extension were carried out in RESOLVE (Terwilliger, 2000). Subsequent model building/rebuilding were performed in the programs "O" (Jones et al., 1991) and COOT (Emsley and Cowtan, 2004). Model refinement was done using CNS (Brunger et al., 1998) and Phenix (Adams et al., 2002) by setting aside 5% of the observed reflection data for cross-validation. The structure was refined to 2.9 Å resolution. More than 88% of the residues are within the favored regions of the Ramachandran plot, ca. 11% within the allowed region (Table 1).

2.2.1. DNA binding assays

0.1 μl of 5 μMol ^{32}P labelled 39 bp DNA was added to 8.9 μl DNA binding buffer (25 nM Tris–acetate, pH 8.0, 20 mM NaCl, 1 mM DTT, 10% glycerol). Wild-type and mutant proteins were diluted in binding buffer to desired concentrations and 1 μl of the diluted protein sample was added to the DNA. The mix was incubated for 1 h on ice before 1 μl 6× DNA loading buffer (40% sucrose and 0.25% bromophenol blue) was added. Samples were then loaded onto a 4.5% native polyacrylamide gel and run at a constant voltage of 80 V for 15 min. The gel was transferred to Whatman paper and dried and imaged using a PhosphoImager system (Fujifilm Image-FLA 500 system). Images were analyzed using the AidaAnalyzer software v3.28 and density was measured using AidaAnalyzer 2D densitometry tool.

3. Results and discussion

3.1. Crystal structure of MexZ

In this work, we present the crystal structure of MexZ at 2.9 Å resolution. MexZ crystallized in space group H32 with one mono-

Table 1
Crystallographic data and refinement statistics.

Crystals	MexZ native		MexZ SeMet	
Data process				
Wavelength (Å)	0.9757	0.9791(peak)	0.9793(inflection)	0.9757(remote)
Space group	H32	H32	H32	H32
Cell parameters				
a (Å)	177.1	176.5	176.9	177.1
b (Å)	177.1	176.5	176.9	177.1
c (Å)	54.7	52.7	52.8	53.0
Resolution (Å)	2.9	3.56	3.55	3.70
R_{sym} (%)[a]	5.7(27.2)	15.5(43.2)	14.8(41.0)	16.2(45.2)
$I/s\langle I\rangle$	5.7(2.6)	7.8(1.5)	8.6(2.2)	6.2(1.8)
Completeness (%)	99.5	91.8	93.8	97.3
Multiplicity	6.1	5.4	5.5	5.6
Beamline	Diamond I0.4	ESRF ID29	ESRF ID29	ESRF ID29
Refinement				
R_{work} (%)[b]	22.65			
R_{free} (%)[c]	27.68			
RMSD bond length (Å)	0.009			
RMSD bond angle (°)	1.729			
Number of protein atoms	1539			
Ramachandran (%)				
Most favored	88.6%			
Additionally allowed	11.4%			
Disallowed	0%			

[a] $R_{sym} = \sum|I_i - \langle I\rangle|/\sum|I|$, where I_i is the intensity of the *i*th measurement, and $\langle I\rangle$ is the mean intensity for that reflection.
[b] $R_{work} = \sum|F_{obs} - F_{calc}|/\sum|F_{obs}|$, where F_{calc} and F_{obs} are the calculated and observed structure factor amplitudes, respectively.
[c] R_{free} = as for R_{work}, but for 10% of the total reflections chosen at random and omitted from refinement.