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Structural analysis of a phosphonate hydroxylase with an access tunnel at the back of the active site

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FrbJ is a member of the Fe^{2+}/α -ketoglutarate-dependent dioxygenase family which hydroxylates the natural product FR-900098 of *Streptomyces rubellomurinus*, yielding the phosphonate antibiotic FR-33289. Here, the crystal structure of FrbJ, which shows structural homology to taurine dioxygenase (TauD), a key member of the same family, is reported. Unlike other members of the family, FrbJ has an unusual lid structure which consists of two β -strands with a long loop between them. To investigate the role of this lid motif, a molecular-dynamics simulation was performed with the FrbJ structure. The molecular-dynamics simulation analysis implies that the lid-loop region is highly flexible, which is consistent with the fact that FrbJ has a relatively broad spectrum of substrates with different lengths. Interestingly, an access tunnel is found at the back of the active site which connects the putative binding site of α -ketoglutarate to the solvent outside.

1. Introduction

The *frbJ* gene was first discovered in a study of the biosynthetic gene cluster of the phosphonate antibiotic FR-900098 in *Streptomyces rubellomurinus* (Eliot *et al.*, 2008). It is a member of the Fe^{2+}/α -ketoglutarate-dependent dioxygenase family. The members of this family are widely distributed in nature, being found in fungi, plants, bacteria and vertebrates. They have diverse sequences and substrate specificities. The most common activity of this group of enzymes is the hydroxylation of an inactivated carbon. Other activities include peroxidation, halogenation and cyclization, dealkylation, ring expansion and contraction reactions *etc.* Owing to their diverse activities, they are involved in many important physiological processes such as DNA repair, xenobiotic degradation, hypoxic response and the biosynthesis of various compounds, including clavulanic acid, cephalosporin and fosfomycin (Hausinger, 2004).

Although the *frbJ* gene was originally discovered in the FR-900098 biosynthesis cluster, it was subsequently proven not to be essential for the synthesis of FR-900098. Instead, it uses FR-900098 as a substrate and catalyzes a hydroxylation reaction yielding another phosphonate antibiotic, FR-33289 (Johannes *et al.*, 2010; Fig. 1). It was later found that FrbJ has a broad substrate spectrum. It also catalyzes the hydroxylation reactions of other substrates, including fosmidomycin, methyl-fosmidomycin and methyl-FR-900098. The products of these reactions were used to construct a library of potential anti-malarial compounds, which have shown good inhibition effects towards the malarial drug target *Plasmodium falciparum* Dxr (pfDxr; DeSieno *et al.*, 2011).

Sequence analysis shows that a group of enzymes called taurine: α -ketoglutarate dioxygenases (TauDs), which catalyze the hydroxylation of taurine and the concomitant conversion of α -ketoglutarate to succinate (Eichhorn *et al.*, 1997), are close homologues of FrbJ. As a model for this enzyme family, the structure and mechanism of TauD from *Escherichia coli* have been studied in great detail (Elkins *et al.*, 2002; Nam *et al.*, 2014; Grzyska *et al.*, 2005; Knauer *et al.*, 2012; Usharani *et al.*, 2011). TauD catalyzes a reaction in which the amino acid taurine (2-aminoethane-1-sulfonic acid) is converted to aminoacetaldehyde and sulfite. Hydroxylation of the inactivated C atom at the α position leads to an unstable intermediate which spontaneously decomposes to the products. TauD bears the signature of the Fe^{2+} / α -ketoglutarate-dependent enzyme family: a facial triad containing two His residues and one carboxylate residue. This motif has the consensus sequence His-Xxx-(Asp/Glu)-(Xxx)_n-His, which is responsible for the coordination of the mononuclear iron (Hausinger, 2004). The structure of TauD contains a jelly-roll motif at the centre and six α -helices surrounding it. The jelly-roll motif lies at the one end of the facial triad containing His99, Asp101 and His255, where the pentacoordinate ferrous iron binds. The cofactor α -ketoglutarate binds to the iron ion in a bidentate manner. The 2-oxo group and C1 carboxylate group coordinate to Fe, while the C5 carboxylate group and Arg266 form a salt bridge. Also present in the active site is the substrate taurine, which forms hydrogen bonds to the amino acids surrounding it. The sulfate group of taurine points to the centre of the binding site, while the amino group points outwards. The sulfate group is tightly packed by the binding amino acids, while the amino group is partially exposed to the solvent outside (Elkins *et al.*, 2002). Another well studied example of this protein family is the alkylsulfatase AtsK from *Pseudomonas putida* S-313 (Müller *et al.*, 2004, 2005). The crystal structure of AtsK indicates that it has a very similar fold to TauD. It forms a tetramer in the crystal structure, which has an inner cavity that is accessible to all four active sites in the tetrameric arrangement. A lid motif was proposed to cover the opening of active site, which would prevent the enzyme from undesired side reactions. Such a lid could be involved in substrate recognition. It may also help to quench the unwanted activated oxygen species formed in the absence of substrate (Müller *et al.*, 2004).

Here, we report the structure of the phosphonate hydroxylase FrbJ. Structural analysis indicates that FrbJ has a jelly-roll core motif at the centre and helical bundle motifs at the periphery. Although its structural composition is similar to other members of the Fe^{2+} / α -ketoglutarate-dependent

Table 1

Macromolecule production.

Source organism	<i>S. rubellomurinus</i>
DNA source	N/A
Forward primer	N/A
Reverse primer	N/A
Cloning vector	N/A
Expression vector	pET-28a(+)
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	MGSSHHHHHSSGLVPRGSHMVEILKKPVTGRSV-WQRAQVEDASQWTVVLDEGMRAEILEAERIN-EQGLTVWDLDRKAVPLERAGKLVACQVEQLEH-GFGLAMLRGVPTTEGLTVAESQVVMGVVGLHLG-TAVAQNGHGDRVVSIRDYKGRLNSKTIIRGYQ-TNESLPWHSADAPDIAALLCLTQAKHGGEFHVA-SAMHIYNTLLQEAPPELLGLYYAGVFFDYRGEE-PPGEPPAYRNAIFGYHNGQLSCRYFLRNFDAS-GTAKLGFQPEVEKLALDTFEEIASRPNHVS-MRLEPGDMLVDNVTVHRRGAYSDEEDGSTD-SSRHLRLWINVENGRQFPSTSLSTRHWGMKAA-AKPTH

dioxygenase family, the orientation of the active-site lid is markedly different, which may suggest different substrate-recognition patterns. An unexpected access tunnel is also found at the rear side of the active site, which implies a different cofactor-transport mechanism of this enzyme.

2. Materials and methods

2.1. Protein expression and purification

The *frbJ* gene was cloned into pET-28a(+) vector and transformed into *E. coli* BL21 (DE3). The recombinant strain was incubated in Luria–Bertani (LB) broth with 50 $\mu\text{g ml}^{-1}$ kanamycin and was induced with 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) when the optical density at 600 nm (OD_{600}) reached 0.6. Incubation continued for an additional 16 h at 16°C before the cells were collected by centrifugation at 6000g at 4°C for 10 min. The resulting cell pellet was resuspended in 30 ml buffer consisting of 20 mM Tris–HCl pH 8.3, 500 mM KCl. The cells were lysed by sonication and the supernatant was collected by centrifugation at 16 000g and 4°C for 30 min.

The supernatant was applied onto Ni–NTA resin (GE Healthcare) pre-equilibrated with NTA-0 buffer consisting of 20 mM Tris–HCl pH 8.3, 500 mM KCl, 10% (v/v) glycerol. The resin was then washed with pre-equilibration buffer supplemented with 30 mM imidazole. After elution with pre-equilibration buffer supplemented with 200 mM imidazole, the fractions were collected and the His tag was removed with thrombin (1 unit per milligram, Sigma). The protein was

Figure 1
The hydroxylation reaction catalyzed by FrbJ.

Table 2
Crystallization.

Method	Vapour diffusion
Plate type	Sitting drop
Temperature (K)	289
Protein concentration (mg ml ⁻¹)	15
Buffer composition of protein solution	20 mM Tris-HCl pH 8.3, 500 mM KCl, 10% (v/v) glycerol
Composition of reservoir solution	100 mM bis-tris pH 6.5, 200 mM NaCl, 25% (w/v) PEG 3350
Volume and ratio of drop	2 µl (1:1)
Volume of reservoir (µl)	120

dialyzed with pre-equilibration buffer and applied onto a Superdex 75 16/60 column (GE Healthcare) equilibrated with Tris-HCl buffer [20 mM Tris-HCl pH 8.3, 200 mM KCl, 10% (v/v) glycerol]. The purified protein was concentrated using 10K Amicon Ultra-4 centrifugal filter units before the concentration was estimated spectrometrically from the OD₂₈₀.

Selenomethionine-labelled FrbJ was expressed as described in the literature and was purified as described for the native protein (Doublié, 2007). In brief, a 2 ml overnight culture was collected by centrifugation at 6000g for 2 min and washed twice with double-distilled water; the cells were resuspended in 1 ml M9 medium and inoculated into 500 ml M9 medium supplemented with 5 g l⁻¹ glucose, 2 mM MgSO₄, 0.1 mM CaCl₂, 2 mg l⁻¹ thiamine, 2 mg l⁻¹ biotin and 50 µg l⁻¹ kanamycin. The culture was grown at 37°C until the OD₆₀₀ reached 0.6. Leucine, isoleucine, valine and L-selenomethionine were added at 50 mg l⁻¹ and lysine, phenylalanine and threonine were added at 100 mg l⁻¹. After incubation for 30 min at 37°C, 0.1 mM IPTG was added to induce protein expression and incubation was continued for a further 16 h at 16°C. Macromolecule-production information is summarized in Table 1.

2.2. Crystallization and data collection

Crystals of native FrbJ were grown by the sitting-drop vapour-diffusion method. 1 µl FrbJ solution (15 mg ml⁻¹) was mixed with 1 µl precipitant solution [0.1 M bis-tris pH 6.5, 0.2 M NaCl, 25% (w/v) polyethylene glycol (PEG) 3350]. The crystals of FrbJ appeared after overnight incubation at 16°C and reached their maximum size 3 d later. Selenomethionine-labelled FrbJ crystals were grown in the same manner. They were soaked in a cryoprotectant consisting of the precipitant solution and 25% (v/v) glycerol before being cooled in liquid nitrogen. Crystallization information is summarized in Table 2.

Diffraction data were collected from selenomethionine-labelled FrbJ crystals at 100 K at the selenium absorption edge using an ADSC Quantum 315 CCD detector on beamline BL17U, Shanghai Synchrotron Radiation Facility (SSRF), Shanghai, People's Republic of China at a wavelength of 0.9791 Å. The crystal-to-detector distance was 250 mm. A total of 245 frames of data were collected with a 1° oscillation range. The data set for FrbJ was indexed, integrated with *MOSFLM* and scaled with *SCALA* from the *CCP4* suite (Winn *et al.*, 2011). Initial phases were obtained by single-wavelength anomalous diffraction (SAD) using the anom-

Table 3
Data-collection and refinement statistics for FrbJ.

Values in parentheses are for the highest resolution shell.

Data collection	
Space group	P2 ₁ 2 ₁ 2 ₁
Unit-cell parameters (Å, °)	<i>a</i> = 53.88, <i>b</i> = 95.09, <i>c</i> = 138.24, α = β = γ = 90.00
Resolution (Å)	50.2–2.3 (2.382–2.300)
Completeness (%)	99.14 (99.94)
Multiplicity	1.9 (1.9)
Anomalous completeness (%)	95.91 (99.72)
Anomalous multiplicity	3.3 (3.7)
<i>R</i> _{merge}	0.0728 (0.3287)
<i>I</i> /σ(<i>I</i>)	8.03 (2.34)
CC _{1/2}	0.990 (0.843)
CC*	0.998 (0.956)
Wilson <i>B</i> factor (Å ²)	33.78
FOM/DM FOM†	0.74/0.89
Refinement	
Total No. of reflections	61592 (6182)
No. of unique reflections	32101 (3200)
<i>R</i> _{work} ‡	0.2186 (0.2875)
<i>R</i> _{free} ‡	0.2412 (0.3080)
No. of non-H atoms	
Protein	4243
Ligands	2
Water	208
Average <i>B</i> factor (Å ²)	
Protein	54.40
Ligands	43.80
Water	58.70
R.m.s. deviations	
Bond lengths (Å)	0.005
Bond angles (°)	0.90
Ramachandran plot§	
Allowed region (%)	2
Favoured region (%)	98
Outliers (%)	0.00
PDB code	5eqn

† Mean figure of merit with/without density modification. ‡ *R* factor = $\sum_{hkl} ||F_{obs}| - |F_{calc}|| / \sum_{hkl} |F_{obs}|$, where *F*_{obs} and *F*_{calc} are the observed and calculated structure factors, respectively. *R*_{free} was calculated with a randomly selected 5% subset of reflections which were excluded from the refinement process. § The statistics of the Ramachandran plot were calculated with *MolProbity*.

alous scattering caused by the selenomethionine incorporated at the methionine sites. The selenium sites in the asymmetric unit were located with the *PHENIX AutoSol* wizard (Adams *et al.*, 2010). The resulting electron-density map was of very good quality and the side chains of 80% of the residues could be automatically built with the *PHENIX AutoBuild* wizard (Adams *et al.*, 2010). Manual building was performed with *Coot* and the structure was refined with *phenix.refine* after each cycle of building (Emsley & Cowtan, 2004). The stereochemistry of the model was validated with *MolProbity* (Chen *et al.*, 2010). The graphics were generated with *PyMOL* (v1.8; Schrödinger).

2.3. Molecular-dynamics simulation

Molecular-dynamics simulation of FrbJ was carried out using the GPU-accelerated *pmemd* code of *AMBER* 14 (Salomon-Ferrer *et al.*, 2013). The ff14SB force field was used to generate the coordinate and topology files of the protein. The *tleap* module of *Amber Tools* 14 was used to add explicit H atoms to the X-ray crystal structure (Case *et al.*, 2015). The system was neutralized by adding Na⁺ counter-ions using *tleap*.

The protein was solvated in a rectangular box filled with TIP3P water molecules (Jorgensen & Madura, 1983). A buffer distance of 15 Å was set between the protein edge and the box boundary in all directions. The protein was minimized in two steps, with each step involving 10 000 steps of steepest-descent minimization followed by 10 000 steps of conjugate-gradient minimization. In the first step the system was minimized keeping the protein fixed using positional restraints with a strength of 500 kcal mol⁻¹ Å⁻². In the second step the whole system was minimized without any positional restraints. After minimization the system was heated to 300 K for 200 000 steps. The system was then equilibrated at 300 K over 5 ns. Finally the whole system was subjected to unrestrained molecular-dynamics simulation for 50 ns, saving the trajectory after each 2 ps. During simulation the pressure was kept constant and the temperature was controlled with the Langevin thermostat (101 kPa, 300 K; Zwanzig, 1973). Long-range electrostatic interactions were computed by employing the particle mesh Ewald (PME) method with the default settings in *AMBER* 14 (Darden *et al.*, 1993; Essmann *et al.*, 1995). The cutoff distances for the long-range electrostatic and van der Waals interactions were set to 10.0 Å. The *SHAKE* algorithm was used for covalent bonds involving H atoms (Ryckaert *et al.*, 1977).

2.4. Access-tunnel prediction

The *CAVER* program was used to identify the access tunnel from the active site to the protein exterior (Chovancova *et al.*, 2012). We chose amino-acid residues in the active site of FrbJ (His150, Asp152, His288, Arg290 and Arg309) as the starting point. The minimum probe radius was set at 1.0 Å, the shell depth was 10, the clustering threshold was 3.5, the maximum distance was 3 Å, the shell radius was 3 and the desired radius was 5 Å. The default values were used for the other parameters.

Figure 2

The overall structure of FrbJ. α -Helices, β -strands and loops are coloured cyan, magenta and brown, respectively.

3. Results and discussion

3.1. The overall structure of FrbJ

The crystals of FrbJ belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 53.88$, $b = 95.09$, $c = 138.24$ Å, $\alpha = \beta = \gamma = 90.00^\circ$. The structure of FrbJ was solved using the selenomethionine-labelled crystals. Data parameters and refinement statistics are summarized in Table 3. There are two molecules in each asymmetric unit. Each FrbJ molecule contains 339 amino acids. Although the structure was resolved for the majority of the amino acids, the regions encompassing residues 126–147, 165–169, 291–305 and 314–339 were missing in both chains, indicating that these residues are flexible. The function of FrbJ indicates that it is a member of the α -keto-glutarate-dependent dioxygenase family. Like other members of this family, a jelly-roll motif formed by residues from both the N-terminal and C-terminal regions is present at the centre

Figure 3

The structural features of FrbJ. (a) The active-site cavity. The conserved His150, Asp152, His288, Arg290 and Arg309 residues, which are involved in substrate, cofactor and metal-ion binding, are shown as cylinders. The rest of the molecule is shown as ribbons. (b) Dimerization interface. The two chains forming the FrbJ dimer are coloured green and cyan, respectively. A stable dimerization interface is found between these two chains (Krissinel & Henrick, 2007).

of the FrbJ structure (Fig. 2). Surrounding the jelly-roll motif are two helical bundles. One is formed by residues from the N-terminal region and the other is formed by residues from the C-terminal region. Each FrbJ molecule contains 14 β -strands and eight α -helices (Fig. 2). Inside the core structure lies the active-site cavity that holds the substrate, cofactor and the metal ion (Fig. 3*a*). At the opening of the jelly-roll motif, the conserved triad (His150, His288 and Asp152), which binds the metal ion, is clearly visible. In addition, two conserved Arg residues (Arg290 and Arg309), which are suggested to bind the α -ketoglutarate cofactor, are located nearby. Interestingly, at the bottom of the active site, a conserved Arg, which acts as the binding site for α -ketoglutarate in the TauD- α -ketoglutarate complex structure, is replaced by a Gln (Fig. 3*a*).

3.2. The dimer interface

Two protomers are found in the asymmetric unit of the FrbJ crystal (Fig. 3*b*). A PISA assay indicates that these two protomers may form a stable dimer in solution with a buried

surface of 1870 Å² (Krissinel & Henrick, 2007). The dimeric interfaces encompass helix 5, helix 1 and the loop at the N-terminus. The interactions between these two protomers are achieved through hydrogen bonding between polar residues on the interfaces. An extensive hydrogen-bond network is also found on the surface of the protein and across the interfaces. This network would also contribute to the stability of the dimer.

3.3. Comparison with the TauD hydroxylase

The FrbJ and TauD structures are homologous in the jelly-roll region, while the structural elements surrounding the core structures are significantly different (Fig. 4). At the opening of the jelly roll lies the lid motif, which is formed by two consecutive helices in TauD (Fig. 4*a*). This lid motif has been shown to undergo structural changes during the binding of tartrate in TauD, which switches the active site from the closed to the open state. In both the closed and the open states of the TauD

Figure 4
Comparison of the lid motifs of FrbJ and TauD. (a) The lid motif of TauD is coloured red. (b) The lid motif of FrbJ is coloured red. The lid motif switches the active site from the closed to the open state. In TauD the active site is partially shielded by the lid motif, but in FrbJ the active site is wide open.

Figure 5
Molecular-dynamics simulation of FrbJ. (a) R.m.s.d. plot of C α atoms: the root-mean-square deviation (r.m.s.d.) of the backbone atoms was calculated relative to the starting model. The simulation converged after 15 ns. (b) Plot of the r.m.s.f. of C α atoms during a 50 ns simulation. The highlighted area shows the significant fluctuation in the lid region.

structure the active site is partially shielded by the lid motif. However, in the structure of FrbJ the lid motif does not consist of helices (Fig. 4*b*). Instead, it consists of two β -strands with a long loop between them. The lid in FrbJ is significantly shorter than that in TauD and it points away from the active site, leaving the active site open. The lid conformation is stabilized by an extensive hydrogen-bond network within the lid structure. It is also strengthened by hydrogen bonds between the lid and helix 7. As indicated by the TauD–substrate complex structure, the lid structure is involved in substrate recognition. The distinct orientations of the lid motifs suggest different substrate-recognition patterns for FrbJ and TauD. It is noteworthy that FrbJ accepts a relatively broad spectrum of substrates with different lengths. Also, more importantly, the hydroxylation site is at the β position in the FrbJ-catalyzed reaction. In contrast, the hydroxylation site is at the α position in the TauD-based reaction. The difference in the character-

istics of these two enzymes may be attributed to the disparity in their active-site configurations.

3.4. Molecular-dynamics simulation analysis

The portions of FrbJ which were absent from the crystal structure were constructed with the *SWISS-MODEL* server. The molecular-dynamics simulation was performed with the full-length FrbJ structure model. To investigate the flexibility of the lid motif, the root-mean-square deviation (r.m.s.d.) with reference to the starting structure was calculated. The r.m.s.d. of the system along the entire MD trajectory is shown in Fig. 5(*a*). The graph shows that the r.m.s.d. of the system converged after 15 ns, which implies that the protein structure is stable. To further understand the internal dynamics of the protein, the root-mean-square fluctuation (r.m.s.f.) of the C^α atoms was computed (Fig. 5*b*). The highest peak is found around the lid section encompassing Asp200–Ala212, which implies that the lid region is highly flexible in the protein (Fig. 5*b*).

Analysis of the MD trajectory using *VMD* shows that the loop from Asp200 to Ala212 fluctuates significantly between two extreme positions (Fig. 6*a*). The protein coordinates from the MD trajectory were extracted at different time points and were aligned using *PyMOL*. Alignment of the structures

Figure 6

The movement of the lid region during the simulation. (*a*) Fluctuation of the lid region. The starting structure is shown in yellow and the final structure after MD simulation is shown in red. The extreme positions are shown in blue and green. (*b*) The alignment of coordinates extracted from the MD trajectory at different intervals. The highlighted portion shows the fluctuating loop.

Figure 7

The predicted access tunnel in the FrbJ structure. The access tunnel is represented by a blue block in the ribbon structure of FrbJ. The residues around the access tunnel are coloured red. The structure of TauD (PDB entry 1gy9; cyan) was overlapped with that of FrbJ (in green) to show the location of the active site.

showed a clear fluctuation in a loop containing residues Asp200–Ala212 (Fig. 6*b*). As the length of this loop is short compared with the full length of the protein, it does not affect the r.m.s.d. of the system significantly.

3.5. The access tunnel at the back of the active site

The access tunnel in the protein active site was predicted with CAVER using the full-length FrbJ structural model (Fig. 7). To our surprise, no tunnel was found at the entrance to the active site. The entrance is partially blocked by Arg290 and Arg309. Instead, an access tunnel was found at the back of the active site. The tunnel is formed by sections encompassing residues 158–172, 272–277, 289–293 and 306–310. At the end of the tunnel are Arg290 and Arg309, which presumably form the binding site for α -ketoglutarate. To our knowledge, this is the first tunnel to be reported for the Fe²⁺/ α -ketoglutarate-dependent dioxygenase family. It has previously been assumed that both α -ketoglutarate and the substrate access the active site through the same entrance, with α -ketoglutarate binding first. Before the substrate binds, the α -ketoglutarate could still be consumed and harmful radicals could be generated. The predicted tunnel at the back of the active site could act as a separate access point for α -ketoglutarate, so that the substrate and cofactor can access the active site simultaneously, which could limit the unproductive consumption of the cofactor. A mutagenesis assay is under way to investigate the function of this access tunnel.

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