The crystal and solution structure of YdiE from *Escherichia coli*

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Iron-containing porphyrins are essential for all life as electron carriers. Since iron is poorly available in an oxidizing environment, bacterial growth may be restricted by iron limitation, and this has led to the evolution of a huge variety of iron-uptake systems. Among pathogens, iron scavenging from the haemoglobin of an animal host is a common means of acquiring sufficient iron for growth. The Isd system of *Staphylococcus aureus* is a well studied example; the bacterium devotes considerable resources to the construction of surface proteins that deftly remove haem from haemoglobin and pass it along a chain of related proteins, eventually delivering the haem to the cytoplasm, where it can be utilized or degraded. All organisms, however, must deal with haem and related molecules, which are by their nature hydrophobic and prone to precipitate, and which tend to promote the formation of reactive oxygen species. Chaperones are an obvious solution to the problem of maintaining a pool of haem for insertion into cytochromes without allowing naked haem to cause damage. YdiE is a very small protein from *Escherichia coli* of only 63 residues which may play a role in haem trafficking. Here, NMR analysis and the crystal structure of the protein to high resolution are reported.

1. Introduction

Although bacteria are frequently labelled as ‘pathogenic’ or ‘nonpathogenic’, the genetic differences between such groups may be very small. The name *Escherichia coli*, for instance, is a broad categorization of many subtypes, some classified by the type of disease they provoke and others known to be harmless commensals. Even attenuated laboratory strains of *E. coli* may carry genes associated with widely feared pathogens. For example, biofilm formation by the plague bacterium *Yersinia pestis* requires the *hmsHFRS* operon, which is highly homologous to *E. coli* *pgaABCD*. The *pgaABCD* operon is fully functional in *E. coli*, even if it does not have the same role as *hmsHFRS* in bacterial transmission between hosts (Bobrov *et al.*, 2008; Darby, 2008). In a similar fashion, the YdiE protein of *E. coli* is closely related to the haemin-uptake protein HemP of *Yersinia* species. These proteins are short, with about 50% sequence identity across the C-terminal half of the protein (comparing *E. coli* and *Y. pestis*). *ydiE* is mentioned directly in only two papers as an ORF regulated by *fur* (McHugh *et al.*, 2003; Panina *et al.*, 2001). No structural or functional characterization of YdiE has been reported. An NMR model of HemP from *Rhodospirillum rubrum* has been deposited in the PDB (PDB entry 2jra; Northeast Structural Genomics Consortium, unpublished work), but no accompanying report has been published to date. Pfam lists 837

Table 1

Data-collection and refinement statistics.

Values in parentheses are for the outer shell.

Data collection	
Space group	$P2_12_12_1$
Wavelength (Å)	0.9000
Unit-cell parameters (Å)	$a = 36.7, b = 38.2, c = 54.7$
Resolution range (Å)	50.0–1.5 (1.55–1.50)
Reflections (measured/unique)	55903/12736
Completeness (%)	98.4 (95.8)
$R_{\text{merge}}^{\dagger}$ (%)	7.3 (31.7)
Overall multiplicity	4.4
Mean $\langle I/\sigma(I) \rangle$	19.3 (4.1)
Refinement statistics	
Resolution range	30.0–1.5
R factor/free R factor	0.171/0.250
R.m.s.d., bond lengths (Å)/angles (°)	0.023/2.06
No. of water molecules	45
Average B factor (protein/water) (Å ²)	15.5/27.8
Ramachandran plot	
No. of residues in favoured regions	77
No. of residues in allowed regions	1

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an observation, $\langle I(hkl) \rangle$ is the mean value for that reflection and the summations are over all equivalents.

sequences of the HemP family from 806 species, suggesting that these proteins are widespread and possibly have a conserved function. Since HemP of *Yersinia* is part of an operon required for TonB-dependent haemin uptake, it seems possible that YdiE is a haem-binding protein.

Apart from the intrinsic interest in haem handling among bacteria and other organisms from a chemical point of view, haem-transfer proteins may provide clues to novel antibacterial strategies by blocking haem-incorporation mechanisms, although YdiE is not an essential protein in *E. coli*. (Malaria parasites are forced to handle excess haem released by the degradation of host haemoglobin by depositing it as an unreactive, insoluble high-molecular-weight complex called haemozoin, and this process has also raised interest as a possible drug target.) We have therefore solved the crystal structure of YdiE, the first protein from its group, and one of the smallest proteins produced by *E. coli*.

2. Materials and methods

2.1. Cloning

ydiE was amplified by PCR from *E. coli* JM109 chromosomal DNA and cloned into a modified pET-28 expression vector using NdeI and XhoI restriction sites. The primer sequences were TTTGCTTCATATGCGTTATACGGATAG-CAGAAAACCTCACGC and TTTGCTCTCGAGTTACTTGGTCAACAGCAGCTTGCCAGCCTG. The PCR product was digested with NdeI and XhoI at 37°C for 2 h before purification using a QIAquick PCR Purification Kit (Qiagen). The purified PCR product was ligated into the cut vector using T4 DNA ligase (Wako) at room temperature for 1 h. The ligation mixture was used to transform *E. coli* DH5 α cells, and pET-28-*ydiE* was prepared from cultures using QIAprep (Qiagen). This construct directs the expression of full-length

YdiE with a hexahistidine tag at the N-terminus that is cleavable with thrombin.

2.2. Expression and purification

pET-28-*ydiE* was transformed into *E. coli* BL21(DE3) cells and the cells were grown at 300 K with shaking in 3 l LB medium containing kanamycin (50 $\mu\text{g ml}^{-1}$). When the OD₆₀₀ of the culture reached 0.6, protein expression was induced by adding IPTG to a final concentration of 1 mM and growth was continued for 3 h at 300 K. The cells were collected by centrifugation at 3000g at 297 K for 30 min. The pellet was suspended in 20 mM Tris-HCl pH 8.50, 0.5 M NaCl, 5 mM imidazole, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM benzamidine and then lysed by sonication on ice. The lysate was centrifuged at 38 000g at 297 K for 30 min. The supernatant solution was loaded onto a 10 ml nickel Sepharose column (GE Healthcare) equilibrated with 50 mM Tris-HCl pH 8.0, 0.3 M NaCl, 10 mM imidazole and, after washing, was eluted with 50 mM Tris-HCl pH 8.0, 0.3 M NaCl, 250 mM imidazole. The major protein fractions were collected and digested with thrombin for 1 h on ice. The protease:YdiE ratio was 1:50. The protein was reloaded onto the washed nickel Sepharose column and eluted with 50 mM Tris-HCl pH 8.0, 0.3 M NaCl. The protein was then loaded onto a HiTrap Q HP column equilibrated with 20 mM Tris-HCl pH 7.5, 1 mM EDTA and eluted with a salt gradient to 1 M NaCl. The pooled fractions containing YdiE were dialyzed into 10 mM Tris-HCl pH 7.5 before concentrating to 22 mg ml⁻¹ using Amicon centrifugal filter units (Millipore). Uniformly ¹³C/¹⁵N-labelled and ¹⁵N-labelled proteins were obtained by growing the bacteria in M9 minimal medium containing ¹⁵NH₄Cl (1 g per litre) either with or without [¹³C₆]-D-glucose (2 g per litre) as the sole carbon source. For the expression of isotope-labelled proteins, the cells were grown for an additional 6 h after IPTG addition.

2.3. NMR analysis of YdiE

Experiments were performed at 298 K using a Bruker DRX600 spectrometer equipped with a triple-resonance Z-axis gradient cryoprobe. The backbone ¹H^N, ¹³C α , ¹³C β' and ¹⁵N and side-chain ¹³C β resonance assignments for ¹³C/¹⁵N-labelled YdiE were obtained from four three-dimensional triple-resonance NMR experiments: CBCA(CO)NH, CBCANH, HNCO and HN(CA)CO. The NMR spectra were processed on Linux PCs with the Azara software package (W. Boucher; <http://www2.ccpn.ac.uk/azara/>). The spectra were analyzed with the ANSIG software (Kraulis, 1989) together with customized programs.

2.4. Crystallization and structure determination

Crystallization experiments were performed at 293 K using the hanging-drop vapour-diffusion method. Crystals grew in 1.6 M ammonium sulfate, 0.1 M Tris-HCl pH 7.0. Data were collected on beamline 38B1 at SPring-8, Harima. The data were collected from a cryocooled crystal which had been soaked briefly in mother liquor with 25%(w/v) glycerol as a

cryoprotectant. A total of 150 images of 1° oscillation were collected. Data processing and scaling were carried out with *HKL-2000* and *SCALEPACK* (Otwinowski & Minor, 1997). The space group was found to be $P2_12_12_1$, with a dimer in the asymmetric unit. Data statistics are given in Table 1. Attempts to phase the structure by standard methods were hampered by the failure to find a heavy-atom derivative and the lack of methionine residues which could be replaced by selenomethionine for anomalous phasing. Replacing leucines with methionines for this purpose resulted in very poor protein expression.

Phases were determined using a combined procedure of homology modelling, molecular dynamics (MD) and molecular replacement (MR). To find structural templates for MR, the sequence of YdiE was compared with the PDB using *BLAST* (Altschul *et al.*, 1997). The best-scoring template structure was the hypothetical protein TSTM1273 from *Salmonella typhimurium* (PDB entry 2loj; Northeast Structural Genomics Consortium/Ontario Centre for Structural Proteomics/Center for Structural Genomics of Infectious Diseases, unpublished work). However, structural analysis of this unpublished NMR structure revealed a significant area of exposed hydrophobic side chains. Naïve molecular replacement with *Phaser* (McCoy *et al.*, 2007) using 2loj as a template was unsuccessful. A second protein with lower sequence identity to YdiE was preferred as a template for homology

modelling since the hydrophobic contacts are in this case buried within domain-swapped dimeric interface. This protein is HemP from *R. palustris* (Rp-HemP); this structure has also been solved by NMR and deposited in the PDB (entry 2jra), but to date remains unpublished.

The homology model was created using the homology-modelling module of *MOE* implemented with the AMBER99 force field (Hornak *et al.*, 2006). Owing to the absence of structural elements in the N-terminal region (residues 1–20), this area was removed from the template. Furthermore, although residues 21–30 are ordered as an antiparallel β -plate in the Rp-HemP NMR structure, these residues are very different from the YdiE structure according to the alignment and were therefore removed as well. Attempts to solve the phases with PDB entry 2jra failed, and therefore a 50 ns molecular-dynamics simulation to sample the conformation was performed using *GROMACS* (Berendsen *et al.*, 1995). Simulation setup and sample extraction were performed according to previously described parameters (Venken *et al.*, 2011, 2012).

Models from the molecular-dynamics trajectory were used as templates for MR with *Phaser* (McCoy *et al.*, 2007). Of 180 models selected sequentially from the MD trajectory for MR, 60 models gave translation-function Z-scores (TFZ scores) of greater than 6.0. The model with the highest TFZ score (8.4) was found to be the correct solution. Model building was

Figure 1

The final structure of YdiE. (a) A stereoview of the C α trace of the YdiE dimer with α -helices shown as coils and β -strands as arrows. One monomer is coloured green and the other blue. The conserved residues His44 and Tyr49 are shown as sticks. The r.m.s.d. for the C α atoms of residues 29–63 in the two monomers is 0.95 Å. The figure was drawn using *PyMOL* (DeLano, 2002). Secondary structure was determined automatically. (b) A stereoview of the final $2mF_o - DF_c$ electron-density map contoured at 1σ .

[illegible]

carried out with *Coot* (Emsley & Cowtan, 2004; Emsley *et al.*, 2010). Refinement was carried out with *PHENIX* (Adams *et al.*, 2010) and *REFMAC* (Murshudov *et al.*, 2011), which is part of the *CCP4* suite (Winn *et al.*, 2011). Noncrystallographic symmetry restraints and TLS-group refinement were not applied. Isotropic temperature factors were refined with default restraints. The Ramachandran plot showed no outliers of any kind, and the agreement between the two copies of the molecule is very close; only Arg26 shows a shift in the plot from a right-handed to a left-handed helix. Figures were prepared with *PyMOL* (DeLano, 2002). Water molecules were checked manually, and two sulfate ions were included at positions showing density with a clear tetrahedral shape. The final model and structure factors have been deposited in the Protein Data Bank as entry 4ynx.

N-terminus of the polypeptide are not visible in the electron-density map, and the value of the Matthews coefficient ($1.3 \text{ \AA}^3 \text{ Da}^{-1}$) suggests that at least some of these are not present owing to natural proteolytic cleavage (Matthews, 1968). The reason for the instability of the Leu→Met mutants is clear from examination of the final structure: the leucine residues play key roles in the highly hydrophobic dimer interface (Fig. 1*b*). A search for related models in the PDB using *DALI* (Holm & Rosenström, 2010) yielded a best match to the initial search model PDB entry 2jra and also a weak match to PDB entry 2loj, but no other models.

3.2. HemP

The Rp-HemP model in the PDB (PDB entry 2jra) shows a domain-swapped dimer, with each polypeptide having a small

3. Results

3.1. Overall structure

Sequence analysis of the *ydiE* gene from *E. coli* showed that the protein has no signal peptide or other regions that are predicted to be unfolded, so a construct was made to express full-length YdiE by PCR from *E. coli* genomic DNA. This construct yielded 19 mg of purified protein per litre of culture (with the N-terminal histidine tag removed). Crystals were grown that diffracted to 1.5 Å resolution, but phasing proved to be problematic as wild-type YdiE has no cysteine or methionine residues apart from the initiator methionine, and only two histidine residues. Phasing was initially attempted by mutating the protein by replacing leucine residues with methionines, but this drastically reduced the protein expression level so that the incorporation of selenomethionine proved impossible. The structure, shown in Fig. 1, was eventually solved by a combined procedure of homology modelling, molecular dynamics and molecular replacement. The starting model was HemP from *R. palustris* (Rp-HemP) solved by NMR (PDB entry 2jra). Two tightly associated copies of the molecule are found in the asymmetric unit. The ordered residues visible in the electron-density map begin at Pro24 and end at Lys63, the C-terminus. Roughly 20 residues at the

Figure 3

The two-dimensional ^1H - ^{15}N HSQC spectrum of YdiE. Cross-peaks are labelled with their corresponding backbone assignments.

Figure 4

A comparison of the crystal and solution structures. (a) A sequence alignment of YdiE with the secondary-structural elements shown above. The possible haem-binding residues His44 and Tyr49 are indicated with blue triangles. RPA2121 is an alternative name for Rp-HemP. This figure was made with *ESPrnt* (Gouet *et al.*, 1999). (b) The secondary-structure assignment from the chemical shift index (CSI). The consensus CSI (CSI_{ave}) for YdiE was calculated according to the following equation: $CSI_{ave} = (CSI^{13}C^{\alpha} + CSI^{13}C^{\gamma} - CSI^{13}C^{\beta})/3$. The secondary-structure assignments agree exactly with the crystal structure.

β -sheet and a single α -helix; two β -strands, one from each partner subunit, form an antiparallel sheet adjacent to the helices. The first 20 residues of each partner are completely disordered. YdiE is slightly smaller than Rp-HemP, but the crystal structure refined to high resolution shows a very similar organization overall. YdiE lacks the initial β -strands of Rp-HemP. Although TSTM1273 has a similar sequence to Rp-HemP, this NMR-derived model (PDB entry 2loj) includes only a monomer, whereas the crystal structure of YdiE is clearly dimeric, with an interface strongly related to that of Rp-HemP. This monomeric structure seems highly unlikely in solution as it exposes a highly hydrophobic surface and analytical ultracentrifugation shows YdiE to be a tight dimer (data not shown). A comparison of the ordered parts of YdiE and Rp-HemP is shown in Fig. 2.

3.3. NMR analysis

It was not clear from the crystal structure whether the N-terminal region of YdiE is ordered or not, so NMR analysis was carried out to determine the secondary structure. The HSQC spectrum is shown in Fig. 3. Backbone $^1H^N$, $^{13}C^{\alpha}$, $^{13}C^{\gamma}$ and ^{15}N and side-chain $^{13}C^{\beta}$ resonances were assigned for all but 15 of the 63 residues. Most of the residues lacking assignments are in the N-terminal region or immediately before the β -strands. Exchange broadening is seen in the HSQC data for the cross-peaks owing to Asn16, Gln31, Gln23, Gln47 and Gln55. The chemical shift index (CSI) method (Wishart & Sykes, 1994) using $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and $^{13}C^{\gamma}$ chemical shifts shows excellent agreement with the crystal structure over the ordered parts of the crystal model (Fig. 4). No loss of N-terminal residues was observed in solution.

4. Discussion

There is a very considerable amount of literature on haem-binding proteins from bacteria, largely because many such proteins are involved in iron scavenging by pathogens and such systems are of great medical interest. Iron restriction is a general mechanism by which a host may inhibit bacterial growth; to counter this, pathogenic bacteria have evolved a variety of receptors and transporters to extract haem from host proteins, which represent an abundant source of haem and iron (Otto *et al.*, 1992; Wandersman & Delepelaire, 2012). Haem-uptake systems are by no means restricted to pathogens, however, and a study of TonB-dependent transporters in marine bacteria suggests that a number of these are dedicated to haem or iron acquisition (Tang *et al.*, 2012). Many lactic acid bacteria such as *Lactococcus lactis* do not produce haem but acquire it from exogenous sources. The first molecular characterization of haem uptake in bacteria was carried out by Stojiljkovic & Hantke (1992). This identified the TonB-dependent haemin-uptake system of *Y. enterocolitica* and demonstrated that HemP, HemR and HemS were necessary for growth under iron-limiting conditions when genes essential for haem- and siderophore-synthesis pathways were mutated (Stojiljkovic & Hantke, 1992). HemS prevents haem toxicity by degrading haem to release the iron (Stojiljkovic & Hantke, 1994). HemR is a receptor that is strongly related (23% sequence identity) to the *E. coli* BtuB protein, a vitamin B₁₂ transporter protein (Heller & Kadner, 1985). The role of HemP was not determined, and the existence of this protein was only inferred from analysis of the DNA sequence downstream from the promoter; *hemP* lies upstream of *hemR*, with the expression of both being regulated by *fur* (Stojiljkovic *et al.*, 1994). The NMR structure of HemP from *R. palustris* has been studied and found to be a domain-swapped dimer, but to

Figure 5

Model quality and the probability of success for MR. The quality of models generated by MD and their TFZ scores when used as templates for MR are compared. The model quality is measured by the C^α r.m.s.d. of each model to the native structure. Model 0 is the homology model used to start the MD simulation. Models 1–180 are selected sequentially from a 50 ns MD trajectory with an equal time step. The models generated have r.m.s.d.s from the native structures ranging from 1.31 to 1.98 Å. The TFZ scores range from 3.6 to 8.4.

date no publication has accompanied the deposition in the Protein Data Bank. In this paper, we have shown that YdiE from *E. coli* has a highly similar structure, with a relatively large, unfolded N-terminal region and a C-terminal region that dimerizes through a highly hydrophobic interface. This C-terminal region is highly conserved across bacterial species and may act as a general haem chaperone. The disordered N-terminal region of the YdiE family is not conserved, and may make specific interactions with other proteins such as HemR encoded by the same operon. Haem binding apparently involves significant structural change, and further work is required to determine the nature of any haem complex. His44 and Tyr49 are the only side chains that might coordinate the haem Fe atom.

The MD simulation played an important role in solution of the YdiE structure, not through refinement of the homology model but rather through the sampling of various conformations of the search model. The initial homology model has an r.m.s.d. of 1.69 Å from the native structure and gave a TFZ score of 4.0. The final structure after MD simulation has an r.m.s.d. of 1.67 Å and gave a TFZ score of 4.6. An intermediate model during the MD simulation (model 75) gave the highest TFZ score of 8.4, but not the lowest r.m.s.d. to the final model (1.56 Å). In fact there is little correlation between the TFZ score and the final r.m.s.d. (as shown in Fig. 5), underlying the need to sample various models during the MD trajectory for successful molecular replacement.

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