PROTEIN STRUCTURE REPORT

Structural characterization of GntR/HutC family signaling domain

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Abstract

The crystal structure of *Escherichia coli* PhnF C-terminal domain (C-PhnF) was solved at 1.7 Å resolution by the single wavelength anomalous dispersion (SAD) method. The PhnF protein belongs to the HutC subfamily of the large GntR transcriptional regulator family. Members of this family share similar N-terminal DNA-binding domains, but are divided into four subfamilies according to their heterogenic C-terminal domains, which are involved in effector binding and oligomerization. The C-PhnF structure provides for the first time the scaffold of this domain for the HutC subfamily, which covers about 31% of GntR-like regulators. The structure represents a mixture of α -helices and β -strands, with a six-stranded antiparallel β -sheet at the core. C-PhnF monomers form a dimer by establishing interdomain eight-strand β -sheets that include core antiparallel and N-terminal two-strand parallel β -sheets from each monomer. C-PhnF shares strong structural similarity with the chorismate lyase fold, which features a buried active site locked behind two helix-turn-helix loops. The structural comparison of the C-PhnF and UbiC proteins allows us to propose that a similar site in the PhnF structure is adapted for effector binding.

Keywords: effector binding domain; transcriptional regulator; HutC; PhnF; chorismate lyase fold; GntR family

A protein containing a sensor input domain and a functional output domain is described as a one-component system (Ulrich et al. 2005). Signal transduction in prokaryotes is

dominated by such systems, where small molecule-binding domains constitute the majority of the input domains and helix-turn-helix (HTH) DNA-binding domains are the most common output domains (Ulrich et al. 2005). Based on sequence similarity, predominantly in the DNA binding domain one-component systems identified in sequenced genomes are assembled into protein families usually named after the best-characterized member. Some of the largest such families (Perez-Rueda et al. 2004) are the LysR (Henikoff et al. 1988), TetR (Ramos et al. 2005), IclR (Nasser et al. 1994), GntR (Haydon and Guest 1991), and AraC (Gallegos et al. 1997) superfamilies. With extensive sequence similarity in the DNA-binding domain, these large families often contain proteins with different types of the effector domains

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Abbreviations: C-PhnF, C-terminal domain of PhnF; C-P, carbon-phosphate; DB, DNA binding domain; HTH, helix-turn-helix; SAD, single wavelength anomalous dispersion; Pn, Phosphonates; TEV protease, Tobacco Etch Virus protease; wHTH, winged helix-turn-helix.

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fused to similar DNA-binding domains (Rosinski and Atchley 1999; Perez-Rueda and Collado-Vides 2000).

This is true for the GntR family of transcriptional regulators, which gathers close to 2000 members in both bacterial and archaeal genomes (Haydon and Guest 1991; Rigali et al. 2002). The proteins in the GntR family share a characteristic version of the N-terminal winged helix-turn-helix (wHTH) DNA-binding domain. This output domain is coupled with the C-terminal signaling domain responding to a range of stimuli in the form of different small molecules. Recent sequence analysis of the GntR proteins revealed the presence of several distinct groups with different types of the C-terminal signaling domains (Rigali et al. 2002). According to this analysis, the GntR family has been divided into four major subfamilies, FadR, HutC, MocR, and YtrA, where each subfamily is categorized by a specific type of the C-terminal domain. While the structure of FadR alone and in complex with its effector and operator DNA has been recently determined (van Aalten et al. 2000, 2001; Xu et al. 2001), no structural information is available for the other three subfamilies of GntR-like regulators.

The HutC subfamily represents more than 30% of all GntR members. It had been named after the HutC regulator from *Pseudomonas putida*, which represses the expression of histidine utilization genes and is, in turn, inactivated by the binding of uroconate (Allison and Phillips 1990). Other characterized members of the HutC family include FarR from *Escherichia coli*, which regulates the expression of citric acid cycle genes and responds to long-chain fatty acids (Quail et al. 1994), the trehalose operon repressor TreR from *Bacillus subtilis* that is inhibited by trehalose-6-phosphate (Schock and Dahl 1996), and a number of proteins (KorSA, KorA, and TraR) that are repressors of the genes involved in

conjugative plasmid transfer in *Streptomyces* species (Kendall and Cohen 1988; Hagege et al. 1993; Kataoka et al. 1994). PhnF from *Escherichia coli*, which is the focus of this article, belongs to the *phn* operon that is involved in transport and biodegradation of phosphonates, (Pn)-compounds having carbon-phosphorous (C-P) bonds (Metcalf and Wanner 1993). Based on its sequence similarity to the HutC proteins, PhnF is predicted to regulate the expression of the *phn* genes and respond to alkylphosphonates. However, its specific regulatory function, its ligand, or the operator sites have not yet been established. A project was undertaken aimed at obtaining structural information on the HutC subfamily ligand-binding domain using X-ray crystallography. In order to increase the chance of successful expression, purification, and crystallization of this protein, only the C-terminal ligand-binding domain, defined by bioinformatics analysis, was included.

Results and Discussion

Our goal was to functionally and structurally characterize the input domain of the HutC subfamily of GntR regulators. Multiple sequence alignments of the selected HutC and GntR family members (Rigali et al. 2002), coupled with a secondary structure prediction, was used to define the domain borders in *Escherichia coli* PhnF (Fig. 1). Three versions of the C-terminal domain differing by few amino acids at the N terminus corresponding to residues 81–, 85–, and 88–241 were selected to increase the chances of obtaining a correctly defined subfragment. In each case the first residue of the domain was selected to be a small hydrophilic residue supposedly positioned between the last strand of the DNA binding domain and the first structural element of the ligand-binding domain

Figure 1. Multiple sequence alignment of PhnF against other members of the HutC family and FadR DNA binding domain. The FadR and C-PhnF secondary structure elements with arrows for β -strands and cylinders for α -helices are shown in red and green, respectively. The first residue of each of the three versions (81–, 85–, 88–241) of the C-PhnF domain is indicated with an arrow. The residues weakly conserved across chorismate lyase and HutC families and the residues conserved among PhnF orthologs are boxed.

(Fig. 1). This region was the least conserved among the HutC proteins according to sequence analysis, and should correspond to the linker connecting the two domains. All three constructs were found to produce soluble protein domains of corresponding size (data not shown). These domains were purified and used for structural and functional studies. Only the PhnF domain corresponding to residues 85–241 produced crystals suitable for structure determination. This version of the PhnF C-terminal domain was selected for further studies and called C-PhnF.

The C-PhnF structure was determined by SAD phasing using seleno-methionine derivatized protein crystals, and was refined to a resolution of 1.7 Å (Fig. 2A; Table 1). The C-PhnF domain structure was composed of a six-stranded antiparallel β -sheet, a two-stranded parallel β -sheet, and four short α -helices. The six-stranded antiparallel β -sheet forms the core of the structure and was constructed by two β -hairpins (S2–S4 and S5–S7), which are

linked by an α -hairpin (H2–H3). The α -hairpin (H2–H3) and the two-stranded parallel β -sheet (S1, S8) followed by a loop (L1) clasp the core β -sheet from one side, while helices H1 and H4 are positioned on the other side. The C-PhnF fold showed no similarity with the FadR structure confirming divergence between the FadR and HutC subfamilies of GntR regulators. On the other hand, the C-PhnF fold had the strongest match with the chorismate lyase (UbiC) structure (PDB code 1FW9; Fig. 2A), with a root-mean-square deviation (RMSD) 3.1 Å and z -score of 10.7 over 125 amino acids according to the Dali search (Heger and Holm 2003). A prediction of the structural similarity between the UbiC fold and HutC regulators' C-terminal domains had been recently made using sensitive sequence profile analysis (Aravind and Anantharaman 2003).

Chorismate lyase is a small universally conserved monomeric enzyme that converts chorismate to 4-hydroxybenzoate. The structure of chorismate lyase from *Escherichia*

Figure 2. (A) Ribbon diagram of the C-PhnF (*left*) and UbiC (*right*) structures. The α -helices and β -strands are numbered and colored red and yellow, respectively. The β -D-fructopyranose molecule (blue) and 4-hydroxybenzoate (magenta) are shown as stick figures. (B) Ribbon diagram of the C-PhnF dimer with monomers in yellow and green. The L1 loop, which adopts a different conformation in each monomer, is highlighted in magenta.

Table 1. X-ray data collection and refinement statistics for PhnF

X-ray data	Peak
Space group	P2 ₁
Unit cell (Å ³)	$a = 46.8, b = 51.6, c = 64.0$ $\beta = 103.6$
Resolution (Å)	50–1.7
Wavelength (Å)	0.97944
Intensity ($I/\langle\sigma\rangle I$)	33.1 (2.75)
Completeness (%)	99.9
R/R_{free}	0.187/0.241
Protein atoms (no.)	2621
Water molecules (no.)	327
Ligand atoms (no.)	12
RMSD bond length (Å)	0.025
RMSD bond angles (°)	2.3
Average protein atoms B-factor (Å ²)	24.1
Average water molecules B-factor (Å ²)	28.3
Average ligand B-factor (Å ²)	18.2

coli (UbiC) had been solved with an internally bound product (Gallagher et al. 2001). Its substrate-binding pocket is formed by two HTH loops that clasp the core β -sheet from one side (Fig. 2A). Determination of the C-PhnF structure allowed performing a comparison of these structures in detail.

The superimposable sections of the UbiC and C-PhnF structures are the central β -sheet, the α -hairpin (H2–H3 in C-PhnF) and the α -helix (H1 in C-PhnF) inserted between the first and the second strand of the β -sheet (Fig. 2A), while the N and C termini of these proteins contain elements that cannot be matched. In the C-PhnF structure these elements primarily involve the two β -strands (S1 and S8 in Fig. 2A), which form a two-strand parallel β -sheet and participate in dimerization and connection with the N-terminal DNA binding domain in context of the full-length PhnF.

The C-PhnF domain crystallized as a symmetric dimer with a tight interdomain interface (Fig. 2B). This interface is formed by the association of the two-stranded parallel β -sheet (S1, S8) from one monomer and a six-stranded antiparallel β -sheet (S2–S7) from the other monomer into an eight-stranded β -sheet (Fig. 2B). The intersubunit contacts are mediated by hydrogen bonding between S8 strand of one monomer and S5 strand of the other monomer. The dimer is an expected minimal functional unit for full-length PhnF, as this is a common feature among regulators with wHTH DNA-binding domains in general and characterized GntR family members in particular (Miwa and Fujita 1988; van Aalten et al. 2000; Kalivoda et al. 2003). Dimerization of the C-PhnF domain was confirmed by gel filtration results (for details, see Materials and Methods). Although the absence of the DNA-binding domain in the current structure does not permit full characterization of the PhnF multimerization mechanism, the tight interaction between

the C-PhnF domains and the symmetry of their dimer strongly suggests that observed interactions are relevant in context of the full-length PhnF.

By comparison with characterized GntR regulators, the C-PhnF domain is expected to bind an effector molecule and transfer this signal to the DNA-binding domain. The nature of this effector remains unknown, although the involvement of PhnF in the regulation of genes responsible for uptake and degradation of alkylphosphonates (Metcalf and Wanner 1993) indicates that its stimuli might be an intermediate of alkylphosphonate metabolism. Structural similarity between C-PhnF and UbiC raises the question whether the location of the effector binding pocket in C-PhnF corresponds to the location of the active site in UbiC. None of the amino acids participating in the binding of the reaction product in UbiC are conserved in PhnF, which is not surprising, since PhnF is not expected to bind either chorismate or 4-hydroxybenzoate. On the other hand, using sequence analysis of various chorismate lyase orthologs and HutC family transcription factors Aravind and Anantharaman (2003) determined that there is weak conservation of certain types of amino acids within the PhnF region that aligns with the UbiC active site. In UbiC these conserved residues define the borders of the active site (Fig. 3), suggesting that in the HutC proteins corresponding amino acids might define the borders of the effector-binding pocket. The conserved amino acid types proposed by Aravind and Anantharaman (2003) include a bulky residue in the middle and toward the end of strand 2 (Arg76 and Leu80 in UbiC) and a polar residue in the middle of strand 3 (Thr92 in UbiC) (Fig. 3). In C-PhnF these residues correspond to Leu132, Arg136, and His148, respectively, and in agreement with the above prediction they are positioned in a similar fashion, with their side chains facing the same side of the central β -sheet (Fig. 3). Nevertheless, the potential pocket secured by these residues in the PhnF structure is largely occupied by the Ile146 side chain, which is situated prior to His148 on strand 3 (Fig. 3). In UbiC, the corresponding position is occupied by Gly90, leaving enough space for the reaction product (Fig. 3).

The UbiC active site is buried behind two HTH loops, one of which is conserved in C-PhnF, while the other one is replaced by a much shorter sequence forming the L1 loop (Figs. 2A, 3). Different conformations for this loop were observed in different C-PhnF monomers with a short α -helix formed in this region in one of them (Fig. 2B). This observation together with high B-factor (data not shown) for amino acids 94–99 compared to the rest of C-PhnF polypeptide points to high flexibility of this region, which may be required for the effector binding.

Several residues conserved among known PhnF orthologs such as Leu94, Ser166, Arg181, Val210, and Ser228

Figure 3. Close view of the potential PhnF effector-binding site in comparison with the active site of UbiC. C-PhnF (*left*) and UbiC (*right*) with bound 4-hydroxybenzoate are colored cyan and purple, respectively. The secondary structure elements are represented in ribbons, while side chains of UbiC active site residues and their counterparts in PhnF are highlighted in yellow. The largest cavity in C-PhnF and the active site cavity in UbiC are represented as molecular surface.

outline the cavity proximal to the region corresponding to the UbiC active site (Fig. 3). This cavity is formed by L1, H2–H3 loops, and β -strands 3–8, and is the largest cavity on the C-PhnF surface according to surface calculations by CASTp (Binkowski et al. 2003). Relatively low sequence conservation among PhnF proteins indicates that accumulation of conserved residues in this cavity is of functional relevance.

This structural analysis drove us to the conclusion that the PhnF region corresponding to UbiC active site is an attractive candidate for the effector-binding site, which may also include the large cavity formed on the C-PhnF surface.

Interestingly, the surface of one of the C-PhnF molecules contained electron density that could not be ascribed to the protein (Fig. 2A). β -D-fructopyranose was modeled into this density to facilitate crystallographic analysis, although no exogenous sugars were added during the purification or crystallization. The modeled molecule forms hydrogen bonds with the terminal amines of Arg231 (2.89, 2.81 Å) and the backbone oxygen of Glu200 (2.46 Å). It is unclear if the found molecule has any functional relevance or it is an artifact of purification or the crystallization process.

To conclude, the C-PhnF structure represents another example of a common fold adapted for enzymatic (UbiC-type) as well as regulatory (HutC-type) functions. According to the earlier prediction (Aravind and Anantharaman 2003), both activities arise from a common small molecule-binding domain, which in the case of HutC regulators had been fused to N-terminal DNA-binding domain and tailored to specifically recognize a diverse range of molecules (Allison and Phillips 1990; Quail et al. 1994; Schock and Dahl 1996). Presented data offers the first insight into the molecular mechanisms behind these evolutionary processes, as well as provides the necessary structural scaffold for further biochemical and structural studies of HutC-type transcriptional regulators.

Materials and methods

Protein cloning, expression, and purification

PhnF residues 81–, 85–, 88–241 were amplified from *Escherichia coli* K12 chromosomal DNA and cloned into the T7 expression vector in fusion with an N-terminal six histidine tag followed by a TEV protease recognition/cleavage site. All three versions of PhnF C-terminal domain were expressed, purified, and concentrated according to the standard procedures described previously (Zhang et al. 2002).

Protein crystallization

The C-PhnF domain was crystallized by vapor diffusion in hanging drops by mixing 2 μ L of the protein solution (20 mg/mL) with 2 μ L of a 20% PEG3350, 0.2 M Mg formate, and 50 mM TRIS (pH 8.0) solution and equilibrated at 20°C over 500 μ L of this solution. Crystals, which appeared after 1 d, were flash-frozen in liquid nitrogen prior to data collection.

Data collection, structure determination, and refinement

Diffraction data were collected at the 19ID beamline at the Advanced Photon Source, Argonne National Laboratory. The data set of 360 images complete to 2 Å at 0.97944 Å wavelength and another 180 images for higher resolution up to 1.7 Å at the same wavelength were collected from a single Se-Met-labeled protein crystal. The space group was $P2_1$ with the cell dimensions of $a = 46.8$ Å, $b = 51.6$ Å, $c = 64.0$ Å, and $\beta = 103.6^\circ$ (Table 1). The data were processed and phased using HKL2000 (Otwinowski and Minor 1997). The structure was determined by SAD phasing using SOLVE (Terwilliger and Berendzen 1999), and an initial model was built after density modification using RESOLVE (Terwilliger 2000). The model was improved using ARP/warp (Perrakis et al. 1999) and refined using REFMAC5 (Murshudov et al. 1997) at 1.7 Å resolution. The final R was 0.187 and the R_{free} was 0.241. The refined model has an excellent geometry with no outliers according to PROCHECK (Morris et al. 1992).

Size-exclusion chromatography

FPLC size exclusion chromatography was performed on a Superdex-75 column preequilibrated with 10 mM HEPES (pH 7.5), and 0.5 M NaCl. The column was calibrated with cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), and bovine serum albumin (66 kDa). Fifty microliters of 20 mg/mL C-PhnF protein was centrifuged at 14,000 rpm for 10 min before being injected into the column. Filtration was carried out at 4°C at a flow rate of 0.5 mL/min. The eluted protein was detected by measuring the absorbance at 280 nm.

Accession numbers

Coordinates and restraints have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>) under PDB code 2FA1.

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