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Elucidation of the crystal structure of FabD from the multidrug-resistant bacterium *Acinetobacter baumannii*

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ABSTRACT

Bacterial fatty acid synthesis (FAS) has been extensively studied as a potential target of antimicrobials. In FAS, FabD mediates transacylation of the malonyl group from malonyl-CoA to acyl-carrier protein (ACP). The mounting threat of nosocomial infection by multidrug-resistant *Acinetobacter baumannii* warrants a deeper understanding of its essential cellular mechanisms, which could lead to effective control of this highly competent pathogen. The molecular mechanisms involved in *A. baumannii* FAS are poorly understood, and recent research has suggested that *Pseudomonas aeruginosa*, a closely related nosocomial pathogen of *A. baumannii*, utilizes FAS to produce virulence factors. In this study, we solved the crystal structure of *A. baumannii* FabD (AbFabD) to provide a platform for the development of new antibacterial agents. Analysis of the structure of AbFabD confirmed the presence of highly conserved active site residues among bacterial homologs. Binding constants between AbFabD variants and *A. baumannii* ACP (AbACP) revealed critical conserved residues Lys195 and Lys200 involved in AbACP binding. Computational docking of a potential inhibitor, trifluoperazine, revealed a unique inhibitor-binding pocket near the substrate-binding site. The structural study presented herein will be useful for the structure-based design of potent AbFabD inhibitors.

1. Introduction

Fatty acid synthesis (FAS) is a ubiquitous system in bacteria and is essential for the viability of the organism. The growth of fatty acid chains during FAS occurs through the condensing enzyme β-ketoacyl acyl-carrier protein (ACP) synthase (KAS) via stepwise elongation of two carbon units supplied in the form of malonyl-ACP. To generate malonyl-ACP, a malonyl group is transferred from malonyl-CoA to ACP harboring a pantethine group attached to a conserved serine residue. The transferase responsible for this reaction is FabD, also called malonyl CoA-ACP transacylase, in *Escherichia coli* and belongs to an operon containing FAS enzymes, such as ACP and KAS II [1]. FabD is also a component of polyketide synthases from *Actinomycetes*, in which malonyl groups form mostly aromatic polyketides [2].

FabD is a protein composed of two main domains. The larger domain contains catalytic residues and belongs to the phospholipase A2 domain superfamily [3], inserted within a small domain of the ferredoxin fold [4,5]. In the proposed mechanism for FabD, the Ser-His dyad in the large domain mediates transacylation via a ping pong bi-bi mechanism. An arginine residue near the dyad was found to be essential for recognition of the malonyl group [6]. The small domain appears to be required for ACP binding according to the structure of the vicenistatin acyltransferase VinK [7]. Structures of FabD and related acyltransferases of polyketide synthases have been determined, including the structure of *E. coli* FabD (EcFabD) [5,6]. The crystal structure of acyl-transferase (AT) from Pks13 of *Mycobacterium tuberculosis* reveals its specificity for very long acyl chains [8].

In extensive studies addressing the issue of multidrug resistant pathogens, FAS has been suggested to be a novel antibiotic target [9,10]. For FabD, corytuberine (COT), a type of quinolone alkaloid, has been found to be active against *Helicobacter pylori* FabD (HpFabD) [11]. Juglone (5-hydroxy-1,4-naphthalenedione) also inhibits the activity of HpFabD [12]. These inhibitors exhibit uncompetitive inhibition, which appears to be relevant to the ping pong bi-bi mechanism of FabD. A calmodulin inhibitor, trifluoperazine (TFP), was found to inhibit the growth of the *E. coli* D22 strain and a resistance mutation was mapped to the *fabD* locus [13]. TFP was also found to inhibit the growth of *M. bovis* with a minimum

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inhibitory concentration (MIC) of 10 μM [14].

Acinetobacter baumannii is a widely distributed nosocomial pathogen, and overwhelming increases in multidrug resistant strains have become a global concern. *A. baumannii* ACICU has one FabD homolog (AbFabD) in its genome, which is located next to 3-ketoacyl-ACP reductase and ACP in the genomic context [15] (Fig. S1). AbFabD shares 51% amino acid sequence identity with EcFabD and 43% sequence identity with *Staphylococcus aureus* FabD (SaFabD). HpFabD shares only 34% sequence identity with AbFabD.

In this study, we aimed to elucidate the high-resolution structure of AbFabD to determine the inhibitory mechanisms of the aforementioned inhibitors and contribute to the development of novel inhibitors of *A. baumannii* FAS.

2. Materials and methods

2.1. Protein expression and purification

The open reading frame encoding AbFabD was amplified from the genomic DNA of *A. baumannii*, subcloned into *Nde*I and *Xho*I sites of the pET28a expression vector, and transformed into *E. coli* BL21 (DE3) (Novagen, USA). For expression of AbFabD, the expression host was grown in LB medium at 37 °C until reaching an OD_{600} of 0.8. The temperature was then lowered to 25 °C, and 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce expression. The cells were then further incubated for 15 h, harvested by centrifugation, and stored at −20 °C until use. Cells were thawed and resuspended in 20 mM Tris (pH 8) and 200 mM NaCl and homogenized by sonication. The insoluble fraction was removed by centrifugation, and cleared lysates were loaded onto HiTrap Chelating Sepharose (5 mL; GE Healthcare, USA) and eluted with a gradual increase in imidazole concentration in elution buffer. The peak fractions containing AbFabD were pooled, and the buffer was exchanged with lysis buffer using Amicon filters (Merck). The final protein concentrations were measured using a NanoDrop instrument (Thermos Scientific).

For generation of holo-AbACP, a co-expression system of AbACP and *A. baumannii* holo-ACP synthase (AbAcpS) was used. AbACP and AbAcpS were cloned into multiple cloning site (MCS) 1 and MCS2 of the pETDuet-1 vector, respectively (Novagen). *E. coli* BL21(DE3) cells containing this co-expression vector were grown in LB medium until the OD reached 0.6–0.7 at 37 °C and treated with 0.2 mM IPTG for induction for 4 h. Holo-AbACP was purified by anion exchange chromatography using Q-Sepharose FF (GE Healthcare). Subsequently, Superdex 75 (GE Healthcare) and Source 15Q (GE Healthcare) columns were used for isolation of holo-AbACP against apo-AbACP, as confirmed by nondenaturing polyacrylamide gel electrophoresis (data not shown).

2.2. Crystallization, data collection, and structure determination

AbFabD was crystallized using the sitting drop vapor diffusion method. Initial sparse matrix screening yielded two conditions crystal of AbFabD. The crystallization buffer contained 1.4 M ammonium tartrate dibasic and 0.1 M Tris (pH 8.5). The crystals were hexagonally shaped rods. A crystal was harvested, briefly transferred to cryoprotectant buffer containing 25% (v/v) glycerol and stored in liquid nitrogen. The crystal was mounted on a nylon loop and kept at −173 °C by chilling with nitrogen gas during the data collection to prevent radiation damage (BL-7A; Pohang Accelerator Laboratory, Korea). The diffraction images were processed by HKL2000 for data reduction and scaling (Table 1) [16].

The structure of AbFabD was determined by the molecular replacement method using the coordinates of *Salmonella typhimurium* FabD (PDB IP 3H0P). Initial phases were used to model AbFabD using Coot [17]. Phenix [18] was used to refine the structure iteratively until the final structure with an *R*-factor of 0.216 and free-*R* of 0.258 was obtained (Table 1). The structure factors and coordinates of AbFabD were deposited into the Protein Data Bank (accession code: 5YPV).

Table 1
X-ray diffraction data collection and structure refinement statistics. Values in parentheses are for the outermost shells.

Data collection	
PDB ID	5YPV
Wavelength (Å)	0.9793
Resolution (Å)	29.21−1.67
Space group	$P3_121$
Cell dimensions	a = 67.46, b = 67.46, c = 110.30
R_{merge}	0.064 (0.605)
$I/\sigma I$	74.0 (5.5)
Redundancy	11.3
Number of unique reflections	34650
Completeness (%)	99.9 (100)
Wilson B-factor	25.8
Refinement	
R-factor	0.216
Free-R	0.258
RMSD	
Bond lengths	0.010
Bond angles	1.083
Overall B-factor ($Å^2$)	33.0
Ramachandran plot	
Favored (%)	98.72
Allowed (%)	0.96
Disallowed (%)	0.32

2.3. Docking simulation of inhibitors

Coordinates and restraints of inhibitors were generated by Grade server [19]. AutoDock was used to dock inhibitors to AbFabD [20]. For the generation of grid maps, the whole active site area was enclosed in the map. Map generation parameters were as follows. For docking simulation, a genetic algorithm was used. Prior to docking, the protein was prepared by adding polar hydrogens and Kollman charges. The number of rotatable bonds in the ligand was set to default. The number of genetic algorithm (GA) runs was set to 100. The Lamarckian GA was used for the scoring.

2.4. Isothermal titration calorimetry (ITC)

AbFabD and variants were concentrated to a concentration of 0.183 mM. The AbACP concentration was set to 1.83 mM. All samples were dissolved in a buffer composed of 20 mM Tris (pH 8.0), 100 mM NaCl, and 2 mM dithiothreitol. To measure the binding affinities (K_a) of AbACP to AbFabD and its variants, ITC was conducted using a MicroCal iTC200 calorimeter (GE Healthcare Life Sciences, Pittsburgh, PA, USA) at National Center for Inter-University Research Facilities (Seoul, Korea) and Korean Basic Research Science (Ochang, Korea). In each titration experiment, 3 μL AbACP was injected into AbFabD with a duration of 6 s at intervals of 120 s. In total, 19 injections were made for each experiment. The dilution heat of AbACP was subtracted, and the resulting data were analyzed by MicroCal Origin software.

2.5. Fluorescence binding assay

The binding of FabD inhibitors was investigated by recording the fluorescence emission spectra of FabD as a function of the concentration of inhibitors. Tryptophan fluorescence was quenched by

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