Biochemical and Biophysical Research Communications xxx (2017) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Structural insights into the dimer-tetramer transition of FabI from *Bacillus anthracis*

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ARTICLE INFO

Article history:
Received 11 September 2017
Accepted 15 September 2017
Available online xxx

Keywords:
Bacillus anthracis FabI
Dimeric form
Tetrameric form
Dimer-tetramer transition

ABSTRACT

Enoyl-ACP reductase (ENR, also known as FabI) has received considerable interest as an anti-bacterial target due to its essentiality in fatty acid synthesis. All the FabI structures reported to date, regardless of the organism, are composed of homo-tetramers, except for two structures: *Bacillus cereus* and *Staphylococcus aureus* FabI (*bc*FabI and *sa*FabI, respectively), which have been reported as dimers. However, the reason for the existence of the dimeric form in these organisms and the biological meaning of dimeric and tetrameric forms of FabI are ambiguous. Herein, we report the high-resolution crystal structure of a dimeric form of *Bacillus anthracis* FabI (*ba*FabI) and the crystal structures of tetrameric forms of *ba*FabI in the apo state and in complex with NAD^+ and with NAD^+-triclosan, at 1.7 Å, 1.85 Å, 1.96 Å, and 1.95 Å, respectively. Interestingly, we found that *ba*FabI with a His_6-tag at its C-terminus exists as a dimer, whereas untagged-*ba*FabI exists as a tetramer. The His_6-tag may block the dimer-tetramer transition, since *ba*FabI has relatively short-length amino acids (^{255}LG^{256}) after the 3_{10}-helix η7 compared to those of FabI of other organisms. The dimeric form of *ba*FabI is catalytically inactive, because the α-helix α5 occupies the NADH-binding site. During the process of dimer-tetramer transition, this α5 helix rotates about 55° toward the tetramer interface and the active site is established. Therefore, tetramerization of *ba*FabI is required for cofactor binding and catalytic activity.

1. Introduction

FabI is the final and rate-limiting step enzyme in the Fatty Acid Synthase II (FAS II) system [1]. Owing to basic differences in the fatty acids biosynthetic mechanisms between human (FAS I) and bacteria (FAS II), the FAS II system is a useful target for anti-bacterial drugs [2,3]. Many FabI inhibitors, such as isoniazid [4], diazaboranes [5], triclosan [6], and other small-molecule inhibitors [7–9], have been developed as anti-bacterial agents. Clinical trials with the FabI inhibitors CG400549 [10–12] and AFN-1252 [13,14] have shown that they possess the safety, efficacy, and potency for the treatment of staphylococcal infections.

The FabI structures reported to date in the Protein Data Bank (PDB), regardless of the organism, are all composed of homo-tetramers except for two structures: *Bacillus cereus* FabI (*bc*FabI) (PDB ID: 3OJE) [15] and *Staphylococcus aureus* FabI (*sa*FabI) (PDB ID: 3GNS and 3GNT) [16], which are composed of dimers. Many cytoplasmic and membrane proteins form homo-oligomeric complexes in cells and play roles in the regulation of cellular processes, such as gene expression, activity of enzymes, ion channels, receptors, and cell-cell adhesion [17]. Formation of homo-oligomers can also provide sites for allosteric regulation and generate new ligand-binding sites at the subunit interface(s) to increase specificity and diversity [17,18]. Hence, one question arises: what is the underlying biological significance of dimeric and tetrameric forms of FabI? To answer the question, *Bacillus anthracis* FabI C-terminally fused to His_6-tag (*ba*FabI-His) or lysozyme (*ba*FabI-T4L) were engineered to block dimer-tetramer transition and artificially maintain the dimer form, respectively. Although the dimeric form of *B. cereus* FabI (*bc*FabI), which is 100% identical to *ba*FabI, is present in PDB, many parts of the protein were disordered due to low resolution, 3.07 Å [16]. Herein, we report the high-resolution crystal structure of the dimeric form of *ba*FabI and crystal structures of the tetrameric

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http://dx.doi.org/10.1016/j.bbrc.2017.09.084
0006-291X/

forms of *ba*FabI in the apo-state and in complex with NAD^+ and with NAD^+-triclosan, at 1.7 Å, 1.85 Å, 1.96 Å, and 1.95 Å, respectively. Our data clearly revealed that conformational changes during dimer-tetramer transition result in a rearrangement of the active site into a catalytically competent conformation.

2. Materials and methods

2.1. Cloning and purification of Bacillus anthracis FabI

The full length *B. anthracis* FabI (*ba*FabI) gene was cloned into the *BamH*I/*Sac*I sites of the expression vector, the modified pET21b vector (Novagen). *Escherichia coli* BL21 (DE3) competent cells transformed with the full-length *ba*FabI construct were grown and induced with 0.5 mM IPTG, for 15 h at 18 °C. Cells were, then, resuspended in buffer A [50 mM Tris pH 7.5, 200 mM NaCl, 5 mM β-mercaptoethanol, 5% (w/v) glycerol, 1 mM PMSF]. The cell lysate was cleared by centrifugation at 12,000 rpm, for 1 h, and the supernatant loaded onto a Ni column (HisTrap HP 5 mL, GE Healthcare). The *ba*FabI protein was eluted with buffer B (buffer A without PMSF) containing 500 mM imidazole. Fractions containing *ba*FabI were pooled and incubated overnight with TEV protease. The sample was purified using a heparin column (Hitrap Heparin HP 5 mL, GE Healthcare) and eluted using a linear 0–500 mM NaCl gradient. Following a second Ni column to remove the His_6-tag and uncut protein, *ba*FabI was further purified using a gel filtration column (HiLoad 16/60 superdex 200 prep grade 120 mL, GE Healthcare) and buffer C [20 mM Tris pH 7.5, 150 mM NaCl, 10% (w/v) glycerol].

To produce *ba*FabI-His, the *ba*FabI gene was cloned into the *BamH*I/*Sac*I sites of the modified pET21b vector with a His_6-tag at the C-terminus. Transformation of competent cells, induction of protein expression, and preparation of bacterial lysate was performed as described above. Elution of *ba*FabI-His was performed with buffer B containing 500 mM imidazole, and the protein was further purified using gel filtration and buffer C.

The *ba*FabI-T4L gene was cloned into the *BamH*I/*Sac*I/*Not*I sites of the modified pET21b vector. The *ba*FabI-T4L protein was eluted with buffer B plus 500 mM imidazole. After pooling the fractions containing *ba*FabI-T4L and incubating the pooled sample overnight with TEV protease, *ba*FabI-T4L was further purified using gel filtration and buffer C.

2.2. Enzymatic assay

The enzymatic activity of purified *ba*FabI proteins was determined in 100 mM sodium acetate buffer (pH 6.5) with 4% glycerol in the presence of 400 μM 2-butenoyl coenzyme A (Sigma-Aldrich) and 200 μM NADH. The reaction was initiated by the addition of FabI, which was used at various concentrations, at 30 °C. Changes in NADH absorbance were monitored for 1 h at 340 nm.

2.3. Multi-angle light scattering (MALS) analysis

MALS experiments were performed during size-exclusion chromatography on a TSK-Gel G3000SWXL column (Tosoh) with a MALS detector (DAWN HELEOS-II, Wyatt Technologies) and a differential refractive-index detector (Optilab T-rEX, Wyatt Technologies). The weight-average molar masses were calculated from the elution data, using ASTRA 6 software (Wyatt Technologies).

2.4. Crystallization, data collection, and structure determination

Purified *ba*FabI was incubated for 3 h, at 4 °C, with a 3-fold molar excess of NAD^+ and triclosan. Cocrystallization experiments were set up using the hanging drop vapor diffusion method. Crystals of *ba*FabI in complex with NAD^+ and with NAD^+ and triclosan were prepared in 0.1 M Na-acetate pH 4.5, 2 M ammonium sulfate. Crystals of apo-*ba*FabI were prepared in 0.1 M MES pH 6.0, 20–45% (w/v) pentaerythritol proproxyllyate (5/4 PO/OH), 0.2 M NaCl. Crystals of *ba*FabI-His were prepared in 0.1 M Tris-HCl pH 8.5, 30% PEG4K, 0.2 M $MgCl_2$. Diffraction data of flash-frozen crystals were collected on 5C beamline at Pohang Accelerator Laboratory. Data were processed and scaled using HKL2000 [19]. All structures were solved by molecular replacement using Phaser [20] with *ba*FabI (2QIO) as the initial search model. The model was manually constructed using COOT [21] and refined with PHENIX [22]. Data collection and refinement statistics are provided in Table S1. The coordinates and structure factors of a dimeric form of *ba*FabI and tetrameric forms of *ba*FabI in the apo state and in complex with NAD^+ and with NAD^+-triclosan have been deposited in the PDB with the codes 5YCX, 5YCV, 5YCR, and 5YCS, respectively.

3. Results and discussion

3.1. Crystal structures of dimeric and tetrameric forms of apo-baFabI

Only three crystal structures of FabI from two organisms, *B. cereus* (PDB ID: 3OJE) and *S. aureus* (PDB ID: 3GNS and 3GNT), in PDB have been reported as dimeric forms [15,16]. The structures have two common features: 1) they have relatively short-length amino acids (^{255}LG^{256} in *bc*FabI and ^{255}IK^{256} in *sa*FabI) at the C-terminus after a highly conserved 3_{10}-helix η7 (Fig. 1A) and 2) they have a His_6-tag at the C-terminus. Therefore, we hypothesized that the reason for these proteins to exist as dimers might be because the His_6-tag blocks the dimer-tetramer transition. However, in the case of other organisms, including *E. coli and Mycobacterium tuberculosis*, the structures of FabI exist as tetramers despite the presence of a His_6-tag at the C-terminus (Fig. 1B). Comparing with *ba*FabI or *sa*FabI, *E. coli* FabI (*ec*FabI) has the additional C-terminal residues ^{254}AAMNELELK^{262} after the 3_{10}-helix η7 (Fig. 1A). The formation of the *ec*FabI tetramer does not appear to be affected by the His_6-tag, since the residues are differently oriented, as shown in Fig. 1C and D [23], and could act as a linker. To confirm our hypothesis that tetramerization of *ba*FabI is blocked by the His_6-tag at its C-terminus, we solved the structures of apo-*ba*FabI with (*ba*FabI-His) and without the C-terminal His_6-tag (*ba*FabI-wt) at 1.7 and 1.85 Å, respectively. *ba*FabI-wt existed as a tetramer (Fig. 2A), whereas *ba*FabI-His existed as a dimer with the crystallographic two-fold symmetry-related monomer, molecule A' (Fig. 2D). When both structures were superimposed, steric clashes occurred between the C-terminal regions of both proteins: the additional C-terminal residues $^{+1}$LEHH^{+4} of molecule A′ in *ba*FabI-His and the C-terminal residues ^{255}LG^{256} of molecule D in *ba*FabI-wt (Fig. 2A–C). Therefore, the His_6-tag at the C-terminus blocks the dimer-tetramer transition in *ba*FabI-His.

In the dimeric form, the substrate-binding loop (SBL, 197–203), the second substrate-binding loop (SBL-2, 99–111), and the active site loop (ASL, 150–160), which play a key role in catalytic activity, were disordered (Fig. 2E). The R111-S121 region was unfolded or disordered in the dimeric forms of *ba*FabI and *sa*FabI reported in previous studies [15,16], whereas it formed an α-helical conformation in our dimeric structure, similar to that of tetrameric forms of *ba*FabI. However, compared to tetrameric forms, the α-helix was rotated about 55° toward the NADH-binding site (Fig. 2F). As a result, NADH is unable to bind to the *ba*FabI-His dimer, because its binding site is occupied by the α-helix α5 and parts of SBL-2 (I94-N98) (Fig. 2F). This is consistent with the result of the enzymatic activity (Fig. 3A). Therefore, our data show that the dimeric form of

Please cite this article in press as: H.T. Kim, et al., Structural insights into the dimer-tetramer transition of FabI from *Bacillus anthracis*, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.09.084