Structural Basis for Catalytic and Inhibitory Mechanisms of β-Hydroxyacyl-acyl Carrier Protein Dehydratase (FabZ)*\(^{1,3}\)

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-β-Hydroxyacyl-acyl carrier protein dehydratase (FabZ) is an important enzyme for the elongation cycles of both saturated and unsaturated fatty acid biosynthetic systems in type II fatty acid biosynthesis (FAS II) enzymes involved in FAS II have been looked at as promising antibacterial agent development targets (1–3). The synthesis of fatty acid in vivo includes initiation and elongation phases. During the elongation cycle of FAS II, four consecutive reactions complete the extension of two carbons (4, 5). In the third step of the elongation cycle, the dehydration of β-hydroxy-ACP to trans-2-acyl-ACP is catalyzed by FabA or FabZ. FabA is a bifunctional enzyme and only found in Gram-negative bacteria with its partner, FabB, to participate in the formation of unsaturated fatty acids (6). FabZ has ubiquitous distribution in the FAS II pathway, and it is a primary dehydratase that participates in the elongation cycles of both saturated and unsaturated fatty acid biosynthesis. Accordingly, FabZ presents itself as a suitable, yet unexplored, target for the discovery of compounds effective against pathogenic microbes (5, 7).

So far, although the detailed enzymatic characterization has been performed for the FabZs from Enterococcus faecalis (EfpFabZ) (8, 9), Pseudomonas aeruginosa (PaFabZ) (7), and Plasmodium falciparum (PfFabZ) (2, 5, 10), and the crystal structures of FabZs from P. aeruginosa and P. falciparum have been determined, the structural basis underlying the catalytic mechanism of FabZ still remains unclear (3, 7, 10). However, because these structures were solved without the presence of inhibitor, they provide little insight into the mechanism of FabZ inhibition.

In our work, to uncover the structural basis underlying the catalytic mechanism of FabZ, the crystal structures of both FabZ from Helicobacter pylori (HpFabZ) and its Y100A mutant have been determined, and their catalytic activities and binding characteristics to ACP investigated. To illustrate the inhibitory mechanism of FabZ, two new inhibitors of FabZ were discovered, and their crystal structures in complex with HpFabZ were determined, which has provided the first structural model of the inhibitor-FabZ complex.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant HpFabZ**—The expression, purification, and enzymatic assay of HpFabZ were performed as described previously (4).

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\(^{1}\) The atomic coordinates and structure factors (code 2GLL, 2GLP, 2GLM, and 2GLN) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

\(^{3}\) The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4 and experimental data.

\(^{1}\) The abbreviations used are: FAS II, type II fatty acid biosynthesis system; FabZ, β-hydroxyacyl-ACP dehydratase; r.m.s.d., root mean square deviation; SPR, surface plasmon resonance.
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**SPR Technology-based Binding Assay**—The compounds for the FabZ inhibitor random screening were from the in-house library. The binding affinity assay of inhibitor against HpFabZ in vitro was carried out by using the SPR technology-based Biacore 3000 instrument (Biacore AB, Uppsala, Sweden) for the primary screening (11). Immunobilization of protein to the hydrophilic carboxymethylated dextran matrix of the sensor chip CM5 (Biacore) was performed by the standard primary amine coupling reaction. HpFabZ to be covalently bound to the matrix was dissolved in 10 mM sodium acetate buffer (pH 4.3). Equilibration of the baseline was completed by a continuous flow of HBS-EP running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.005% (v/v) surfactant P20, pH 7.4) through the chip for 1−2 h. Biacore data were collected at 25 °C with HBS-EP as the running buffer at a constant flow of 20 μl/min. The equilibrium dissociation constant (K_D) for evaluation of the protein-ligand binding affinity was determined by the steady-state affinity fitting analysis of the Biacore data.

**Enzymatic Inhibition Assay**—Following the SPR technology-based binding assay, the compounds with high binding affinities to FabZ (K_D < 10 μM) were further validated by enzymatic assay. The enzymatic inhibition assay of HpFabZ was monitored by the spectrophotometric method (4,5). In brief, the activity of HpFabZ was measured by detection of the decrease in absorbance at 260 nm for the conversion of crotonoyl-CoA to β-hydroxybutyryl-CoA. The compound dissolved in 1% Me_2SO was incubated with the enzyme for 1 h before the assay was started. The 50% inhibitory concentration (IC_{50}) of each inhibitor was estimated by fitting the inhibition data to a dose-dependent curve using a logistic derivative equation (11). The inhibition mode assay for HpFabZ was also monitored using the spectrophotometric approach (4,5). The reaction mixture consisted of 10 μg of HpFabZ in 20 mM Tris-HCl at pH 8.0, 500 mM NaCl, 1 mM EDTA, and 1% Me_2SO in a total volume of 200 μl. The decrease in absorption at 260 nm using crotonoyl-CoA as substrate was monitored for 5 min at 25 °C. The inhibitor type was determined in the presence of various inhibitor concentrations (0−50 μM). After 2 h of incubation, the reaction was started by the addition of crotonoyl-CoA (10−250 μM). The K_1 values were obtained from Lineweaver-Burk double-reciprocal plots and subsequent secondary plots.

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. pQE30-HpFabZ plasmid was used as template for construction of the HpFabZ (Y100A) mutant. The mutant was sequenced to confirm the desired mutation site. The expression and purification of HpFabZ (Y100A) were similar to those of HpFabZ as described above. The enzymatic characterization of HpFabZ (Y100A) mutant was determined according to the previously published method (4). The binding affinity of HpFabZ or its mutant to holo-HpACP was performed on the SPR technology-based Biacore 3000 instrument (12), in which the holo-HpACP protein was obtained and immobilized on the CM5 chip as described in our previous work (13).

**Crystallization and Data Collection**—The purified HpFabZ was dialyzed against 20 mM Tris-HCl at pH 9.0, 500 mM NaCl and concentrated to ~10 mg/ml. For crystallization, 1 μl of protein was mixed with an equal volume of reservoir solution containing 2 M sodium formate, 0.1 M sodium acetate trihydrate at pH 3.6−5.6 and benzamidine-HCl was added to a final concentration of 2% (w/v). The mixture was equilibrated against 500 μl of the reservoir solution at 277 K by the hanging-drop vapor-diffusion method. Crystals of dimensions 0.5 × 0.3 × 0.3 mm^3 were obtained after 7 days. Inhibitor was added to the original drop to a final concentration of ~20 mM, and the crystals were soaked for 24 h. Similarly, crystals of HpFabZ (Y100A) mutant in dimensions of 0.3 × 0.3 × 0.1 mm^3 were obtained after 5 days in reservoir solution (2 M sodium formate, 0.1 M sodium acetate trihydrate, pH 4.0−6.2).

Diffraction data were collected at 100 K using CuKα x-ray with a Rigaku R-AXIS IV++ image plate. Before crystals were flash-frozen in liquid nitrogen, the drop was dehydrated against 500 μl of reservoir solution containing 4.0 M sodium formate for 24 h. The data were processed using HKL2000 (14). Analysis of the diffraction data indicated that the native crystals belong to space group P2_1_2_1 and mutant crystals to C2. The crystallographic statistics are summarized in Table 1. The structures were solved by molecular replacement method with the crystal structure of PaFabZ as the search model and refined by using the program CNS (15). Electron density interpretation and model building were performed using the computer graphics program "coot" (16).

**RESULTS**

**Structure of HpFabZ**—The native HpFabZ protein used for structure determination was not truncated. The crystal structure was determined by molecular replacement (MR) to a resolution of 2.2 Å. The final structure of HpFabZ is well ordered, and the electron densities for all residues with the exception of the N terminus (residues 1−10) are clearly interpretable. The statistics of the diffraction data and structure refinement are listed in Table 1. Similar to the structures of PfFabZ (2) and PaFabZ (7), the x-ray crystal structure of the native HpFabZ is determined as a hexameric architecture, displaying a classic “trimer of dimmers” organization (Fig. 1A). The six monomers arrange a ring-like (A-B-F-E-C-D-A) contact topology.

As expected from the homology to PaFabZ, PfFabZ, and EcFabA at the amino acid sequence level (4), the HpFabZ monomer also adopts a typical β_+ + α “hot dog” fold (17,18), where six anti-parallel β-sheets with topology 1/2/4/5/6/3 wrap around a long central six-turn α-helix (α3) located between β2 and β3 (Fig. 1B). Additionally, for HpFabZ, a two-turn α-helix (α1) is located at the N terminus, and a short α-helix (α2) is plugged between β2 and α3. Notably, an extra short two-turn α-helix (α4), never discovered in the structures of any other FabZ or FabA, is found between α3 and β3 (Fig. 1C). Sequence alignment reveals that α4 is formed by the extra residues (residues 87−95) (Fig. 1D), where there is usually a flexible loop or a gap in the structures of EcFabA, PaFabZ, or PfFabZ (supplemental Fig. S1). As will be discussed below, this extra α-helix in HpFabZ plays an important role in shaping the substrate-binding tunnel.

The overall structure of the HpFabZ dimer is similar to the dimers of PaFabZ (7), PfFabZ (2), and EcFabA (17). The HpFabZ dimer is formed mainly via the hydrogen bonds between residues 102−108 of the two β3 strands from two dif-
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TABLE 1
Summary of diffraction data and structure refinement statistics

| Structure       | HpFabZ  | HpFabZ-compound 1 complex | HpFabZ-compound 2 complex | Y100A mutant |
|-----------------|---------|---------------------------|---------------------------|--------------|
| Data collection | P2,2,1  | P2,2,1                     | P2,2,1                     | C2           |
| Space group     |         |                           |                           |              |
| Cell dimensions (Å) |       |                           |                           |              |
| a               | 73.857  | 74.003                    | 74.011                    | 266.525      |
| b               | 100.585 | 100.578                   | 100.408                   | 76.913       |
| c               | 185.752 | 186.472                   | 186.044                   | 113.187      |
| α               | 90.00   | 90.00                     | 90.00                     | 90.00        |
| β               | 90.00   | 90.00                     | 90.00                     | 102.25       |
| γ               | 90.00   | 90.00                     | 90.00                     | 90.00        |
| Wavelength      | 1.5418  | 1.5418                    | 1.5418                    | 1.5418       |
| Resolution (Å)  | 50.00-2.20 (2.28-2.20) | 50.00-2.42 (2.51-2.42) | 50.00-2.60 (2.68-2.60) | 50.00-2.50 (2.59-2.50) |
| Rmerge          | 0.132   | 0.064 (0.270)             | 0.033 (0.077)             | 0.074 (0.285) |
| Completeness (%) | 97.3    | 92.1 (87.9)              | 84.3 (83.3)               | 97.8 (94.6)  |
| Redundancy      | 3.96 (4.29) | 4.60 (4.50)           | 4.40 (4.30)               | 3.30 (3.10)  |

Refinement

| Resolution (Å)  | 50.00-2.20 (2.28-2.20) | 50.00-2.42 (2.51-2.42) | 50.00-2.60 (2.68-2.60) | 50.00-2.50 (2.59-2.50) |
| No. reflections | 71005 (6987)            | 49567 (4663)            | 45511 (4426)            | 76473 (7381) |
| Rwork/Rfree    | 0.214/0.255             | 0.189/0.235             | 0.187/0.241             | 0.240/0.293 |

B-factors

| Protein        | 7268               | 7291                   | 7291                    | 14518       |
| Benzanidine-HCl | 72                 | 90                     | 63                      | 53          |
| Cl ions        | 6                  | 6                      | 6                       | 12          |
| Water molecules| 597                | 538                    | 549                     | 218         |
| Compound       | -                  | 42                     | 33                      | -           |

R.m.s deviations

| Bond lengths (Å) | 0.008 | 0.008 | 0.007 | 0.009 |
| Bond angles (°)  | 1.4   | 1.3   | 1.3   | 1.5   |

* Numbers in parentheses represent statistics in highest resolution shell.
* Rsym = \( \sum_h ||I_h|-<I_h>|/\sum_h I_h \)
* Rwork = \( \sum_{h,i} ||F_h|-|F_i||/\sum_{h,i} F_i \)

Different monomers, thus all the sheets constitute a 12-stranded curved anti-parallel β-sheet layer (Fig. 2A). The dimer is further stabilized by the hydrophobic interactions within residues Val-68, Leu-69, and Val-71 in the last two turns of α3 helix, and interactions between residues Pro-22, His-23, and Phe-59* (the prime indicates the residue from the other subunit in the dimer). The dimer forms two substrate-binding tunnels with catalytic sites on the interface, which are ~20 Å away from each other (Fig. 2A). Each catalytic site is formed by two highly conserved residues, His-58 and Glu-72* (His-49 and Glu-63* in PaFabZ; His-133 and Glu-147* in PfFabZ) that are located in the middle kink of the substrate-binding tunnel made up by the conserved residues nearby (Figs. 1D and 2, B and C). For the tunnel, residues Tyr-100 (Tyr-88 in PaFabZ; Leu-170 in PfFabZ; and Arg-104 in EcFabA), Phe-101 (Phe-89 in PaFabZ; Phe-171 in PfFabZ), Met-102 (Val-90 in PaFabZ; Ala-172 in PfFabZ), Ile-64* (Ile-55* in PaFabZ; Ile-139* in PfFabZ), Phe-109* (Phe-97* in PaFabZ; Trp-179* in PfFabZ) and Pro-112* (Pro-100* in PaFabZ; Pro-182* in PfFabZ) form a 4.5-Å wide entrance, permitting the substrate access to the catalytic site (Fig. 1D). The entrance is near the ACP binding groove (7), which is composed of a floor (residues Tyr-100, Met-102, Thr-103, Ile-132, Met-154, and Ala-156) and an edge (residues Lys-129, Lys-108*, and Arg-110*).

Residue Tyr-100 of each monomer acts as a doorkeeper of the tunnel, residing between the entrance and the ACP groove (Fig. 2, B and C). Remarkably, Tyr-100 adopts two types of conformations in the whole structure of HpFabZ hexamer: Tyr-100 is recognized in a closed conformation in monomers A-C (The average B-factor values of Tyr-100 are 51.479 in chain A, 39.567 in chain B and 34.856 in chain C) (Fig. 2C) and in an open conformation in monomers D–F (The average B-factor values of Tyr-100 are 32.293 in chain D, 30.564 in chain E and 35.967 in chain F) (Fig. 2B). This is quite different from the structures of PaFabZ and PfFabZ, where the corresponding residues were found in an open conformation for only one monomer. In the closed conformation (Fig. 2C), Tyr-100 is stabilized by the Van der Waals interactions with Met-102, Met-154, and Pro-112*. In the open conformation (Fig. 2B), Tyr-100 floppy ~120° around the Cα-Cβ bond and is stabilized by the Van der Waals interactions with Met-102, Lys-62*, and Ile-64*, allowing the chains of substrates to enter the tunnel. In contrast to the high conservation of residues near the entrance, residues near the bottom of the tunnel are not conserved (Fig. 1D). Residues Pro-91, Ile-93, Ala-94, Lys-95, and Thr-96 in the unique α4 helix constitute half of the bottom of the tunnel. Residues Ile-20, Leu-21, Pro-22, Ala-94, Lys-95, and Lys-97 form a “back door (exit)” for the tunnel. Like Tyr-100, Phe-83 (Phe-74 in PaFabZ, Leu-158 in PfFabZ, and Gly-95 in EcFabA) acting as a doorkeeper from the exit also adopts two major conformations, closed and open (Fig. 2, B and C),
which may be associated with the catalytic function of HpFabZ (see discussion below). When the phenyl ring of Phe-83 takes a closed conformation, it points toward Ile-98. In the open conformation, Phe-83 rotates 120° around the Cα-Cβ bond and its side chain points toward Ile-93, exposing the exit to the bulk solvent. Meanwhile, the two conformations of Phe-83 lead the tunnel to form two shapes, L-shape and U-shape, as will be discussed below.

Three dimers interact with each other through hydrogen bonds (H-bonds) and hydrophobic interactions to form a hexamer (Fig. 1A). On the dimer-dimer interface, Thr-49 at the loop between α2 and β2 forms H-bonds with Asp-31 at the N-terminal of α1’ and Lys-46 at the C-terminal of β2’; and Phe-50 hydrophobically interacts with Ile-14, Leu-18, and Leu-28. Because of these strong H-bonds and hydrophobic interactions, HpFabZ still possesses enzymatic activity and high stability at extreme conditions, such as high temperature (>90 °C) and extreme pH value as described in our previous report (4). In addition, the β3 and β6 strands of monomers B and C turned around toward the β5 strand with the largest displacement of 8.8 Å (Fig. 2D), causing the formation of two “grooves” that are ~4 Å wide and ~16 Å long on the surfaces of the dimer A/B and dimer C/D (Fig. 2, E and F), which have never been observed in the structures of PaFabZ, PfFabZ, and EcFabA. As will be discussed below, these grooves facilitate the binding of inhibitor.

The Role of Tyr-100 in the Catalytic Activity of HpFabZ—As mentioned above, Tyr-100 adopts two conformations in the crystal structure (Fig. 2, B and C). To investigate whether the flexibility of Tyr-100 is associated with the function of HpFabZ, site-directed mutagenesis of tyrosine 100 to alanine 100 (Y100A) was performed. The enzymatic assay showed that this mutant possessed less than 50% enzymatic activity of the wild type with $K_m = 197.8 ± 12.6 \mu M$, $k_{cat} = 0.0039 \text{s}^{-1}$, and $k_{cat}/K_m$.

FIGURE 1. Schematic diagram of the HpFabZ overall structure. A, hexamer of HpFabZ. B, monomer (180°-rotated) of HpFabZ. Secondary structural elements are labeled. C, topology diagram of HpFabZ. Helices are drawn as cylinders, and strands are drawn as arrows. The ribbons are drawn by PyMOL (24), and the secondary structures are assigned by the program DSSP (25). D, multiple structure-based sequence alignment of HpFabZ with PaFabZ, PfFabZ, and EcFabA. Secondary structures of HpFabZ are labeled. Identical and similar residues are shaded. The residues around the active tunnel entrance are marked with triangles. The alignment was calculated by the CE algorithm (26) and produced by the program STRAP (27) with slight modification.
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FIGURE 2. Schematic diagram of the HpFabZ dimeric structure. A, dimer (180°-rotated) of HpFabZ. The surfaces of two active tunnels in the dimer are represented, respectively, and the entrance and exit of each tunnel are also marked by arrows. B, side view of HpFabZ active tunnel surface (the residues Phe-83 and Tyr-100 belong to monomer F). The picture was produced by Molcad of the program suite Sybyl.5 Door residues (Phe-83, Tyr-100) and active residues (Glu-72, His-58) are shown as sticks. The tunnel shows a conformation with entrance door opened and exit door closed; thus the substrate chain has to stretch into the bottom of the L-shaped tunnel due to the closing of the exit door. C, side-view of HpFabZ active tunnel surface (the residues Phe-83 and Tyr-100 belong to monomer A). The tunnel possesses another conformation with entrance door closed and exit door opened, then long substrate chain can stretch out from the exit door. D, schematic picture of the superposition between dimer A/B and dimer C/D. β3 and β6 in dimer D as well as β3’ and β6’ in dimer B are labeled. There is an obvious shift (the largest displacement is 8.8 Å) of these two strands (β3 and β3’) between monomer B and D, and the shift is shown with arrows. E, top view of HpFabZ active tunnel surface (dimer A/B, the residue Tyr-100 belongs to monomer B), produced by PyMOL. The tunnel is not covered by the surface, exposing part of its hydrophobic inner wall to the bulk solvent. The door residue Tyr-100 points toward Pro-112 to display a closed conformation. As a result, a new tunnel entrance was formed by residues Ile-98, Ala-100, Phe-101, Met-102, Phe-59, Ile-64, Phe-109, Arg-110, and Pro-112, and this entrance, ~15 Å in width, completely exposed the active site to the bulk solvent (Fig. 3D), indicating that the mutant may be more suitable for ACP binding, because the ACP binding groove of Y100A mutant is widened. To further investigate the role of Tyr-100 in ACP binding, the binding affinities of ACP with both HpFabZ and its Y100A mutant were determined by using the SPR technique-based assay (Fig. 3B). Indeed, the binding affinity of HpFabZ (Y100A) mutant to holo-HpACP was significantly enhanced compared with that of wild-type HpFabZ.

**Discovery of HpFabZ Inhibitor**—The compounds for FabZ inhibitor screening were from the in-house library. In the primary screening, the compound binding affinity assay against FabZ enzyme was carried out by SPR technology-based Biacore instrument. At this cycle, about 10 compounds with high binding affinities to FabZ (K_D < 10 μM) were obtained and further applied to enzymatic inhibition assay, among which 2 compounds (named compound 1 and compound 2 in the article) were discovered to exhibit inhibition against HpFabZ with an IC_{50} value of 39.8 ± 0.35 and 47.6 ± 0.29 μM, respectively. In addition, the competitive inhibition modes for both of these two inhibitors were also determined by K_i values of 9.7 and 4.3 μM, respectively (Fig. 4, C and F), comparable with their K_D values of 8.61 and 7.59 μM produced from the SPR assay (Fig. 4, B and E).

To test whether these two compounds could also inhibit FabZs of other species, the recombinant EcFabZ was cloned and purified (see supplemental data). The enzyme inhibition assay showed that these two HpFabZ inhibitors could also inhibit the enzymatic activity of EcFabZ by IC_{50} values of 32.8 ± 0.65 (compound 1) and 40.4 ± 0.41 μM (compound 2), thus suggesting that these two inhibitors might act as leads for developing antibiotics.

In addition, we have ever also performed the antibacterial assay for these two inhibitors in vivo. However, because of their too poor solubility in medium, no acceptable results could be obtained in the related assays.

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5 Sybyl Molecular Modeling Package (2000) Version 6.8, Tripos Associates, St Louis, MO.
More recently, by screening natural product library, Tasdemir et al. (19) discovered several flavonoids as PfFabZ inhibitors. We also discovered two flavonoids PfFabZ inhibitors (compounds 3 and 4) that exhibit moderate binding affinities and inhibitory activities toward HpFabZ (supplemental Fig. S4), thereby indicating that HpFabZ might act as a potent target for discovering inhibitors against not only *H. pylori* but also other bacteria.

**Figure 3.** Effect of Y100A mutation on biochemical and structural characters of HpFabZ. A, enzymatic activity of HpFabZ and Y100A mutant. B, binding affinity of HpFabZ and Y100A mutant to holo-HpACP. C, top view of the HpFabZ active tunnel surface (the residue Tyr-100 belongs to monomer F). The door residue Tyr-100 displays an opened conformation. D, top view of the active tunnel surface for Y100A mutant (the residue Ala-100 belongs to monomer F). The entrance is enlarged and exposed part of its hydrophobic inner wall to the bulk solvent.

**Figure 4.** Chemical structures of compound 1 (panel A) and compound 2 (panel D); the kinetic analysis of compound 1 (panel B) and compound 2 (panel E) binding to HpFabZ by SPR technology-based Biacore 3000. Representative sensorgrams obtained from injection of compound 1 and 2 at concentrations of 1.18, 1.68, 2.40, 3.43, 4.9, 7, and 10 μM. The mode of inhibition for compounds 1 and 2 are competitive inhibitors for the substrate crotonoyl-CoA. The lines intercepted on the 1/V axis, indicating that both compound 1 (panel C) and 2 (panel F) are competitive inhibitors for the substrate crotonoyl-CoA.
molecules of compound 1 bind to one hexamer of HpFabZ with two distinct interaction models: one molecule fits into the groove around the entrance (Fig. 6A) (designated as model A hereinafter); the other molecule lies at the active site of the tunnel (Fig. 6B) (called model B below). For model A, Tyr-100 and Phe-83 adopt open and closed conformations, respectively, thus the pyridine ring of compound 1 is located between the phenol ring of Tyr-100 and the pyrrolidine ring of Pro-112, forming a sandwich structure; and the 2,4-dihydroxy-3,5-dibromo phenyl ring at the other end of compound 1 binds into a pocket formed by Arg-158, Glu-159, Phe-59', Lys-62, and Tyr-100 via hydrophobic interactions. For model B, the whole molecule of compound 1 entered into the middle of the tunnel that is located near the active site of HpFabZ (His-58 and Glu-72'). The pyridine ring almost accessed the exit of the tunnel, contacting with Ile-20, Leu-21, Pro-22, Phe-83, Ala-94, Lys-97, and Val-99 via hydrophobic interactions. While the 2,4-dihydroxy-3,5-dibromo phenyl ring is sandwiched between Ile-98 and Phe-59', and Tyr-100 adopted a closed conformation and Phe-83 adopted an open conformation.

Unlike compound 1, compound 2 can only bind to monomer B of HpFabZ with a binding model similar to model A and adopt an extended planar conformation to fully fill the groove of the entrance (Figs. 6D and 6C): the phenyl ring is sandwiched between Tyr-100 and Pro-112' through a hydrophobic interaction; the 2-chloro-benzoic acid moiety is stabilized by cation-π interaction with the positive charged carbamidine group of Arg-158 and H-bond interaction with the backbone carbonyl oxygen of Pro-60' (20). The oxygen of the 2-methoxy-ethyl chain of compound 2 also forms H-bond with the backbone nitrogen of Phe-101.

The coordinates and structure factors of HpFabZ, HpFabZ complexed with compound 1, HpFabZ complexed with compound 2, and HpFabZ (Y100A) have been deposited in the Protein Data Bank (PDB accession codes: 2GLL, 2GLP, 2GLM, and 2GLV). The codes of the PDB Chemical Component Dictionary for compounds 1 and 2 are BDE and SCB in the crystal structures.

DISCUSSION

To date, only the crystal structures of PaFabZ and PfFabZ have been determined (2, 7). In this work, the x-ray crystal structure of HpFabZ was determined. Although the overall structure of HpFabZ is similar to those of PaFabZ and PfFabZ (supplemental Fig. S1), the crystal structure of HpFabZ still has its unique features. For the monomer of HpFabZ, an additional α helix (α4) that does not exist in PaFabZ or PfFabZ could be observed between α3 and β3 (Fig. 1D). This extra short two-turn α-helix shapes half of the tunnel bottom (Fig. 2, B and C), which solidifies the tunnel in comparison with the tunnels of PaFabZ and PfFabZ. The crystal structure of HpFabZ also reveals the flexibility of Phe-83, a non-conserved residue at the bottom of the tunnel. When Phe-83 adopts the closed conformation, the exit (back door) of the tunnel is closed and the tunnel is shaped as an L-form (Fig. 2B); when Phe-83 turns out as in the open conformation, the exit is opened up and the tunnel turns into a U-form.
The existence of the two forms of the tunnel in HpFabZ clearly explains the paradox that the tunnel length of FabZ (~18 Å) is not adequate to accommodate longer (>16-carbon acyl chain) substrates (2), for the tails of long substrates may thread the exit. While in EcFabA, this doorkeeper does not exist (the corresponding residue is Gly-95) (Fig. 1D), it can thus only catalyze short substrates (<10-carbon).

Another major difference between the hexamer structure of HpFabZ and those of PaFabZ and PfFabZ is that the former is unsymmetrical and the later are highly symmetrical. For the hexamer structure of HpFabZ, monomers A, D, E, and F fold into an identical conformation, and monomers B and C adopt a different conformation. Structural superposition indicates that the difference between these two conformations comes from the positions of β3 and β6 strands (Fig. 2D). The β3 and β6 strands of monomers B and C turn around toward the β5 strand, the largest displacement being up to 8.8 Å. The exceptional conformations of monomers B and C cause another structural specialty of HpFabZ, i.e. there is a ~4-Å wide and ~16-Å long groove on the surfaces of dimers A/B and C/D.

FIGURE 6. Crystal structures of HpFabZ in complex with compounds 1 and 2. The dimers that bind to the compounds are represented. The hydrophobic surface of the active tunnel and the critical residues that interact with the compounds are also shown.
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This groove, never seen in the structures of PaFabZ and PfFabZ, is located around the entrance of the tunnel. As shown in Fig. 2, E and F, this groove is exactly where the inhibitors reside.

In this work, two different HpFabZ-inhibitor binding models have been proposed. As indicated in the complex structures, the hydrophobic active tunnel is exposed to bulk solvent due to the displacement of β3 and β6 strands in monomers B and C. Such displacement contributed a lot to the hydrophobic interactions between protein and inhibitors. While in the structures of PaFabZ and PfFabZ, six monomers are highly symmetric with a 3-fold axis and no displacement could be observed in β3 strand. The active tunnels of the hexamer are completely blocked by the surface, thus interfering with the inhibitors entry into the active tunnel to form co-crystal. However, such a β3 strand orientation difference between HpFabZ and PaFabZ might be related to crystal packing, although their β3 strands might exhibit much more flexible conformation in solution (2).

Therefore, according to the above-mentioned indication, the determined inhibitors seem to show inhibition activities against HpFabZ, PfFabZ, and EcFabZ as indicated by enzyme assay but fail to form crystals in complex with PfFabZ and EcFabZ. Additionally, the poor solubility of the inhibitors and the weak hydrophobic inhibitor-enzyme interaction may lead to the relatively low occupancy of the inhibitors in the complex structure, thus resulting in a high B-factor. In fact, such a high B-factor issue seems to be common for soaking-approach-based protein/small molecule complex structures (21, 22).

It is noticeable that there are above normal number of water molecules in the structures (over twice as many as in other similar structures), which, we think, could be also acceptable. During the structural analysis, the CNS program (water_pick. inp) was used for water molecule pick up. The sigma cutoff value was set to 3.0. Most of the water molecules had excellent 2Fo-Fc electron densities contoured at 1.25 σ, indicating the accuracy of the picked water molecules. The large number of the water molecules containing in the crystals might be due to the following dehydration approach. In considering that the crystals would be cracked when soaked in the cryoprotectant even with a low concentration of glycerol, the crystal-containing drop was dehydrated against 500 μl of reservoir solution with 4.0 M sodium formate for 24 h before the crystals were flash-frozen in liquid nitrogen. Thus, it seems that our different dehydration approach has caused the difference in the number of water molecules in the crystals.

The x-ray crystal structure of HpFabZ revealed the flexibility of Tyr-100 which adopted two conformations, open and closed conformations, at the entrance of the tunnel (Fig. 2, B and C). Although the flexibility of corresponding residues in PaFabZ (Tyr-88) (7) and PfFabZ (Leu-170) (2) were addressed by the x-ray crystal structure determinations, they have not been appreciated. Our site-directed mutagenesis revealed that the enzymatic activity of HpFabZ (Y100A) mutant decreases less than 50% (Fig. 3A). This highlights that Tyr-100 might facilitate the reaction of fatty acid biosynthesis. The binding data indicates that ACP binds to HpFabZ (Y100A) mutant much stronger than to the wild-type HpFabZ. In particular, the dissociation step of ACP from the HpFabZ mutant is extremely slow as shown in Fig. 3B. This may be the major reason for the enzymatic activity of the mutant droppin less than 50%. After the dehydration of substrate (β-hydroxyacyl-ACP) is completed, the product (trans-2-acyl-ACP) has to exit and vacate the tunnel for the next molecule of α-hydroxyacyl-ACP. Slow dissociation of ACP from FabZ thus slows down the catalytic rate of the enzyme. Indeed, the x-ray structure shows that the entrance of the mutant is enlarged due to the small size of the side chain of substituted Ala-100, which is beneficial to binding with ACP. From these experimental results, we can deduce that the side chain of Tyr-100 may switch frequently between the open and closed conformations (Fig. 2), and the waggling movement of Tyr-100 is an important driving force for products leaving FabZ. Based on the mutagenesis result and x-ray structures, a dynamic model for the catalytic process of FabZ during the biosynthesis of fatty acids can be devised. Firstly, β-hydroxyacyl-ACP binds to HpFabZ with Tyr-100 in an open conformation, and the substrate β-hydroxyacyl-ACP enters into the tunnel for dehydration. After the reaction is finished, Tyr-100 turns to the closed conformation and stimulates the dehydration product trans-2-acyl-ACP to leave FabZ, and the next molecule of β-hydroxyacyl-ACP binds to FabZ for dehydration.

Enzymes encoded in the FAS II pathway have recently been appreciated as promising targets for discovering anti-bacterial and anti-parasitemia (malarial) drugs (3, 8, 23). The currently available FabZ inhibitors are NAS derivatives originally found as EcFabA inhibitors and flavonoids against PaFabZ (5, 19). So far, no inhibitors have been reported for FabZs of other microorganisms. We have discovered four HpFabZ inhibitors through random screening of our in-house chemical library (Fig. 4, A and D and supplemental Fig. S4), and for the first time, we obtained the structures of HpFabZ complexed with two of the inhibitors (compounds 1 and 2). In addition to residing in the grooves on the surface, one molecule of compound 1 has entered into the center of the tunnel. This indicates that the enzymatic activity of HpFabZ can be inhibited via two ways, either occupying the entrance of the tunnel or plugging the tunnel to prevent the substrate from accessing the active site.

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