Crystal structures of bacterial FabH suggest a molecular basis for the substrate specificity of the enzyme

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FabH (β-ketoacyl-acyl carrier protein synthase III) is unique in that it initiates fatty acid biosynthesis, is inhibited by long-chain fatty acids providing means for feedback control of the process, and dictates the fatty acid profile of the organism by virtue of its substrate specificity. We report the crystal structures of bacterial FabH enzymes from four different pathogenic species: Enterococcus faecalis, Haemophilus influenzae, Staphylococcus aureus and Escherichia coli. Structural data on the enzyme from different species show important differences in the architecture of the substrate-binding sites that parallel the inter-species diversity in the substrate specificities of these enzymes.

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1. Introduction

Increased antimicrobial drug resistance in organisms such as methicillin resistant Staphylococcus aureus (MRSA) has necessitated the search for new therapeutic agents against these and other pathogens [1]. One of the keys to overcoming this problem is to identify a compound with a novel mode of action toward a new target enzyme. The bacterial fatty acid biosynthesis (fab) pathway has been shown to be essential for cell growth [2–5] and may provide us with one such target.

All organisms synthesize fatty acids via a conserved cycle of condensation, reduction, dehydration and reduction of carbon–carbon bonds [3], but mammals encode a single multifunctional multi-domain enzyme that hosts the active site for each of the synthetic steps on a different domain. This is referred to as the type I pathway. Bacteria, on the other hand, follow the type II pathway where each enzymatic activity is found on a distinct protein [6]. The protein sequences and the enzymatic active sites of the two types bear little resemblance to each other leading us to believe that it should be possible to design specific and potent inhibitors of enzymes from the type II fab pathway, with little or no cross-reactivity with the type I enzyme [6].

Excellent reviews of the fab cycle can be found elsewhere [3,6]. FabH catalyzed condensation of malonyl–acyl carrier protein (ACP) with acetyl-CoA to form β-ketobutyryl-ACP is the initiation step of straight chain saturated fatty acid biosynthesis. In addition to being the regulatory enzyme for the fab cycle, the fatty acid profile of an organism is determined by the substrate specificity of its FabH enzyme [5,7]. Escherichia coli, wherein straight chain fatty acids are predominant, acetyl-CoA and propionyl-CoA are the preferred substrates for FabH [8]. On the other hand, in Bacillus subtilis and Streptomyces glaucescens, which are known to produce both straight and branched chain fatty acids, FabH can use branched chain acyl-CoA in addition to straight chain acyl-CoA as substrates [7]. The molecular basis for this substrate specificity of the enzyme is not clear though bacterial FabH from various species have been structurally characterized [9–14].

Here we present six structures of FabH enzymes from pathogens Enterococcus faecalis, Haemophilus influenzae, Staphylococcus aureus and E. coli. The analysis of available FabH structures shows subtle differences in the active site architectures of these enzymes from Gram-positive and Gram-negative organisms. These differences parallel the differences seen in the substrate specificities of these enzymes and hence provide a basis for not only explaining experimentally determined substrate specificities, but also provide...
a framework from which the fatty acid profiles of different bacteria can be predicted using the sequence analysis of their FabH enzymes. Two of the structures presented here were determined in complex with novel inhibitors shedding light on the mechanism of molecular recognition. These co-crystal structures identify the interactions between the protein and the compounds that can be potentially developed into high affinity inhibitors using the observed principles of molecular recognition.

2. Materials and methods

2.1. Expression, purification and crystallization of FabH

Full-length E. coli, E. faecalis, S. aureus and H. influenzae FabH proteins were cloned into pET expression vectors (Novagen) with an N-terminal His-tag (pET28) as well as in tagless version (pET30). All proteins were expressed in E. coli strain BL21 (DE3) (Invitrogen). Harvested cells containing his-tagged FabH were lysed by sonication in 20 mM Tris pH 7.6, 5 mM imidazole, 0.5 M NaCl and centrifuged at 20 000 rpm for 30 min. The supernatant was applied to Ni-NTA agarose column (Qiagen), washed, and eluted using a 5–500 mM imidazole gradient over 20 column volumes. Eluted protein was dialyzed against 20 mM Tris pH 7.6, 1 mM DTT, and 100 mM NaCl. Purified FabH were concentrated up to 2 mg/ml and stored at −80 °C in 20 mM Tris pH 7.6, 100 mM NaCl, 1 mM DTT, and 20% glycerol for enzymatic assays or concentrated up to 20 mg/ml for crystallization trials.

Harvested cells expressing tagless FabH were sonicated in 25 mM Tris 8.0, 20 mM NaCl, 2 mM DTT, 1 mM EDTA, 50 mM IME, and 0.5 ml (per 100 ml lysate) protease inhibitor cocktail (Roche). The crude lysate was centrifuged at 20,000 rpm for 30 min and the soluble fraction was separated by anion-exchange chromatography (HiPrep 16/10 Q XL, Amersharm Pharmacia Biotech (APB)) using a 0–1 M NaCl gradient over 10 column volumes. Protein fractions were checked by SDS–PAGE and appropriate fractions were dialyzed against 25 mM Tris 8.0, 2 mM DTT, and 50 mM IME. Dialyzed protein was loaded onto a 10 ml Source Q-15 (APB) and further separated by anion-exchange chromatography using a 0–500 mM NaCl gradient over 20 column volumes. Protein fractions were checked by SDS–PAGE and FabH containing fractions were pooled and loaded onto an 8 ml hydroxyapatite column (BioRad) and eluted with 0–250 mM gradient of a pH 8.0 potassium phosphate buffer over 15 column volumes. FabH containing fractions were concentrated up to 2 mg/ml and stored at −80 °C in 20 mM Tris pH 7.6, 100 mM NaCl, 1 mM DTT, and 20% glycerol for enzymatic assays or concentrated up to 20 mg/ml for crystallization trials.

All crystals were obtained by vapor diffusion using 20 mg/ml protein solution in the hanging drop setups at 15 °C. N-terminally His-tagged E. coli FabH was crystallized with 12%PEG 3350, 5 mM calcium acetate, 0.1 M HEPES, pH 7.5, 2 mM TCEP and 1% DMSO. The CoA bound structure of E. faecalis FabH was obtained using the tagless protein with two point mutations, A192T and N253S, which were cloning artifacts. These mutations have no impact on the tagless protein with two point mutations, A192T and N253S, which were cloning artifacts. These mutations have no impact on the protein structure as inferred from comparison with the compound 13a bound E. faecalis FabH structures obtained with the wild-type sequence. The crystallization condition consisted of 25% PEG 3350, 0.1 M HEPES, 0.125 M magnesium sulfate, and 1.5% DMSO. The co-crystal structure of E. faecalis FabH with bound compound 13a was obtained using the N-terminally His-tagged protein. The crystallographic conditions consisted of 0.2 M lithium sulfate, 0.1 M Bis-Tris, pH 6.5, 25% PEG3350. The structure of E. faecalis FabH with bound compound 23i was determined using the tagless protein with two specific surface mutations (D36A, E37A, which improved crystallizability) crystallized with 25% PEG 3350, 0.2 M ammonium sulfate and 0.1 M Bis–Tris, pH 5.5. H. influenzae FabH structure was obtained using N-terminally tagged protein and crystallization condition consisting of 35% PEG 4000, 0.4 M ammonium acetate, 0.1 M sodium citrate, pH 6 and 15% glycerol. Tagless S. aureus FabH gave crystals with 2 M sodium formate, 0.1 M sodium acetate, pH 4.6.

2.2. Enzyme inhibitors

Biochemical screening of 2500 select compound library resulted in 27 hits with IC50 < 10 μM. Of the four structurally diverse series identified, the benzoylamino-benzoic acid series of compounds was chosen for optimization. Two of these compounds, 2-(4-bromo-3-diethylsulfamoyl-benzoic)benzoic acid (13a) and 2-(4-(3,5-dimethyl-piperidin-1-yl)-3-phenoxymethylamino-l-benzoic acid were synthesized at Quorex as described elsewhere [15]. The compound 13a showed an IC50 of 1.6 μM and 23i an IC50 of 0.27 μM against E. faecalis enzyme.

2.3. Data collection and structure determination

All data were collected at 110 K using MAR image plate and rotating anode X-ray generator. Crystals were frozen in synthetic mother liquor with 25% glycerol. Data were integrated, reduced and scaled using the HKL program suite, CXN was used for molecular replacement and refinement of structures. Structure of the E. coli FabH was first determined by molecular replacement using the 1.8 Å published structure of the same protein (PDB ID: 1EBL)[10] as the starting model. All the subsequent structures were determined by molecular replacement, using E. coli FabH structure as the starting model.

2.3.1. Accession codes

The coordinates for structures solved in this study have been deposited in the Protein Data Bank with ID codes 3IL3, 3IL4, 3IL5, 3IL6, 3IL7 and 3IL9.

3. Results and discussion

3.1. FabH structures

Including the structures reported here (Table 1), we have crystal structures of the FabH enzyme from eight different bacterial species, two of which are Gram-positive organisms (E. faecalis and S. aureus [11]) and five Gram-negative (E. coli [10,12], H. influenzae, A. aerolicus (PDB ID: 2EBD), T. thermophilus (PDB ID: 1UB7) and B. pseudomallei (PDB ID: 3GWE)); M. tuberculosis [13,14] falls under neither Gram classification. As has been noted earlier [12], the FabH monomer has internal pseudo-symmetry in its structure suggestive of a gene duplication event some time during its evolution. Based on this symmetry, the structure can be divided into N-terminal and C-terminal domains. However, there is barely any sequence similarity between the two domains of the same protein. The inter-domain structural divergence between N- and C-terminal domains within the FabH enzyme is most pronounced in the first loop (L1 of the N-terminal domain and L9 of the C-terminal domain), which shows that Cys112 is acetylated with no suggestion of an entrained cysteine sulfinic acid. In contrast, H. influenzae enzyme structure shows that Cys112 is acetylated with no suggestion of an entrained small molecule in the active site.
Fig. 1. Stereo drawings of acetyl CoA recognition by *E. faecalis* FabH. (a) 3 Å resolution $F_o - F_c$ difference electron density map contoured at 3$r$ (blue) and 5$r$ (red) superimposed on the final refined model of acetyl CoA. (b) Details of the acetyl CoA binding.

### Table 1: Crystallographic data and refinement statistics.

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<tr>
<th></th>
<th><em>E. coli</em> FabH</th>
<th><em>E. faecalis</em> FabH in complex with Acetyl CoA 13a</th>
<th><em>H. influenzae</em> FabH</th>
<th><em>S. aureus</em> FabH</th>
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<td>54 436</td>
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<td>$R_{sym}$</td>
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<td>$I/\sigma$</td>
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<td>9.1 (3.1)</td>
<td>11.9 (4.3)</td>
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*Fig. 1.* Stereo drawings of acetyl CoA recognition by *E. faecalis* FabH. (a) 3 Å resolution $F_o - F_c$ difference electron density map contoured at 3$r$ (blue) and 5$r$ (red) superimposed on the final refined model of acetyl CoA. (b) Details of the acetyl CoA binding.
We obtained two co-crystal structures of *E. faecalis* FabH in complex with benzoylamino-benzoic acid derivatives, 2-(4-bromo-3-diethylsulfamoyl-benzoamino)-benzoic acid (13a) and 2-[4-(3,5-dimethyl-piperidin-1-yl)-3-phenoxy-benzoamino]-benzoic acid (23i) (Fig. 2) that show reasonable inhibition of the enzyme [15]. The binding modes of the two are essentially identical, with the compounds binding as far into the active site cleft as physically possible (Fig. 3); they make 3.5 Å van der Waals contact with Cα atom of Gly313 at the cleft floor. The benzoic acid group occupies the deep end of the CoA binding site, in close proximity to the catalytic triad. The phenyl ring of the benzoic acid makes σ–π interaction with Phe312, the residue that plays a significant role in defining the shape of the binding site, and probably the substrate specificity of the enzyme (see below). The amide N of the benzoylamino group makes intra-molecular hydrogen-bonding interaction with the carboxylate of the benzoic acid group. The carbonyl O of the benzoylamino group makes hydrogen-bonding interaction of the backbone N of Phe224. The phenyl ring of the benzoylamino group is facing the solvent exposed entrance.

**Fig. 2.** Benzoylamino-benzoic acid derivatives that inhibit FabH enzymatic activity.

**Fig. 3.** Molecular recognition of the small molecule inhibitors by *E. faecalis* FabH. Some of the interactions between the protein and the compound 13a (a) or 23i (b) are shown. Carboxylate of the benzoic acid group makes hydrogen-bonding contacts with the active site residues His250 and Asn280, shown by the dashes.
of the binding cleft. More detailed discussion of the small molecule interactions with the protein can be found elsewhere [15].

3.2. Cis-peptide bonds

Two residues are found to have cis-peptide bonds in the FabH structures from all four species reported here. These are Pro93 (and its equivalent Pro88 in *S. aureus*, *H. influenzae* and *E. coli*) and Leu316 (Leu302 in *S. aureus*, Trp307 in *H. influenzae* and Phe308 in *E. coli*) of *E. faecalis* protein. These two residues are far apart in the primary sequence and the tertiary structure of a single molecule. However, within the functional dimer, these two cis-peptides from two different molecules are not only brought in the immediate neighborhood of each other by virtue of dimerization, but are also close to, but not in the immediate vicinity of, the active site cleft. Dimerization brings N-terminal sequence neighbor of the cis-Pro (Met92 in *E. faecalis*, Tyr87 in *H. influenzae* and Phe87 in *S. aureus* and *E. coli*) close to the active site Cys, thereby contributing to the formation of the substrate-binding cleft of the dimeric partner.

3.3. Substrate specificities and differences in the substrate-binding sites of FabH from different species

Despite wide range of pairwise sequence similarities (28.9–66.2%), substrate-binding sites of FabH from various species are remarkably well conserved, at least in terms of the sequence (Fig. 4). We have identified 22 residues that make up the large substrate-binding sites of FabH from different species) at the base of the CoA binding cleft. We present strong evidence suggestive of two classes of binding sites based on the type of fatty acids synthesized by the organisms. Side chains Phe312 of *S. aureus* and Tyr304 of *M. tuberculosis* FabH are pointed inside the active site cleft of the enzyme, thereby decreasing the size of the cleft. The rotamer adopted by the Phe/Tyr of the enzyme in Gram-positive organisms is a relatively rare one (frequency of occurrence 13% [16]), and is probably associated with an energetic cost that is likely compensated elsewhere. The Gram-negative organisms *E. coli* and *H. influenzae* on the other hand, have the equivalent Phe side-chains (Phe304 and Phe303, respectively) oriented in the opposite direction so as to be within the hydrophobic core of the protein (Fig. 5). The sidechain rotamer here is a relatively higher frequency (33% occurrence [16]) and hence lower energy one. As a result, these organisms have FabH active site clefts that are significantly bigger than in *M. tuberculosis* or the Gram-positive *E. faecalis* and *S. aureus* FabH. These specific rotamer conformations are forced by the residue that is a layer

![Fig. 4. Sequence analysis of the residues constituting the FabH/ZhuH substrate-binding site. Except the last sequence, which is for *Streptomyces ZhuH* enzyme, all others are for FabH. The residue numbering on the top refers to the *E. faecalis* FabH; the numbering on the bottom refers to the *E. coli* protein. The sequences have been grouped according to the Gram classification of the bacteria: the top group is for Gram-positive, the bottom group is for the Gram-negative organisms; the one in the middle falls between. Residues in gray show some variability, other are largely conserved. The last two residues in the sequences do not contribute to the substrate-binding site. Abbreviations: Efa – *Enterococcus faecalis*; Sau – *Staphylococcus aureus*; Spy – *Streptococcus pyogenes*; Spn – *Streptococcus pneumoniae*; Bps – *Burkholde-
Fig. 5. Illustration of the structural consequences of identities of residues at positions equivalent to Phe226 and Val231 of E. faecalis protein on the rotamer conformation of the equivalent of Phe312, which dramatically influences the pocket size and hence the compound binding. In all (a–d) panels, Phe226, Val231 and Phe312 from E. faecalis FabH are shown in red. The view on the left shows part of bound compound 13i. Each of the four panels shows two orthogonal views. The equivalent residues shown in yellow (and labeled on the right hand panels) are from (a) E. coli, (b) H. influenzae, (c) S. aureus and (d) T. thermophilus FabH. Panel (d) also shows equivalent residues from M. tuberculosis FabH in blue.
removed from the immediate vicinity of the CoA binding site. In *E. faecalis* FabH, this residue is Phe226, which is located right behind Phe312 in the hydrophobic core of the enzyme. The equivalent residue in *S. aureus* is Phe209 and in *M. tuberculosis* Trp215. However in the Gram-negative *E. coli* and *H. influenzae* FabH, the residues (equivalent to Phe226 of *E. faecalis* FabH) are Val215 and Leu214, respectively, which have much smaller side chains. Consequently, not only Phe304 in *E. coli* and Phe303 in *H. influenzae* enzymes have the freedom to orient their side chains in the hydrophobic core, but not doing so would leave a hole in the core of the protein – clearly an energetically expensive proposition. Additionally, another residue lining the CoA binding tunnel, Leu220 in *E. coli* and Leu219 in *H. influenzae* FabH, precludes the Phe in question to take up the rotamer that would point the side chain within the cleft. Corresponding to Leu220 in *E. coli* FabH is the smaller side chain Val231 in *E. faecalis* protein, which does not interfere with Phe312 side chain pointing inside the cleft. Thus, the FabH sequences in *E. faecalis*, *S. aureus* and *M. tuberculosis* on one hand and *E. coli* and *H. influenzae* on the other, dictate significant differences in the shape of the deep end of the substrate-binding site by forcing the individual amino acid side-chains to adopt particular rotamers.

It is evident that the pair of residues at positions equivalent to Phe226 and Val312 in *E. faecalis* protein must determine the rotamer conformation of the residue at the position equivalent to the Phe312, and in the process determine the shape of the active site cleft (Fig. 5). Based on this premise, we looked at the sequences of some of the important Gram-positive and -negative pathogens to see if there is a trend vis-à-vis the Phe residue that changes the shape of the CoA binding site. Interestingly, all Gram-positive organisms we looked at retain the Phe226 of the *E. faecalis* enzyme, leading to the prediction that they must have a CoA binding site that is analogous to the *E. faecalis* FabH, by virtue of the Phe312-equivalent pointed within the binding site (Fig. 4). The FabH sequences from the Gram-negative organisms, however, do not present the same consistent pattern. The substrate-binding site for the Gram-negative *T. thermophilus* FabH clearly resembles that of Gram-positive organisms rather than the binding sites of the Gram-negative *E. coli* and *H. influenzae* FabH. This is consistent with the sequence-structure correlation drawn above for Gram-positive organisms. While the sequences of *Salmonella typhimurium*, *Helicobacter pylori* and *Vibrio cholerae* FabH strongly suggest that their CoA binding clefts have structures very similar to the crystallographically observed sites in *E. coli* and *H. influenzae* FabH, those of *Rickettsia rickettsii*, *Yersinia pestis* and *Neisseria meningitides* suggest that they look more like FabH from other Gram-positive organisms.

Interestingly, the prediction concerning the shape of the substrate-binding pocket of FabH from different bacterial species parallels the experimental evidence that a number of these bacterial species can use branched chain primers in order to produce branched chain fatty acids [17,18]. For example, *S. aureus*, most *Bacillus*, *Streptococcus pneumoniae*, *Listeria monocytogenes* are known to synthesize branched chain fatty acids [17]. *B. subtilis*, *S. glaucescens*, *Streptomyces coelicolor* and *S. pneumoniae* FabH have been shown to be active in the presence of iso-butyryl CoA [5,18,19]. Protein sequences of *S. coelicolor* and *S. glaucescens* clearly suggest that their substrate-binding pockets are akin to the substrate-binding pocket of *E. faecalis* enzyme. On the other hand, *E. coli* FabH is known to be selective for acetyl-CoA, initiating the synthesis of straight chain fatty acids [8,19–21].

This analysis of the binding site also explains the reported differences in cross-genus activity [15] of some of the inhibitors that are analogs of the benzoylarnino-benzoic acid derivatives (Fig. 2). These inhibitors are nearly equipotent against *E. faecalis* and *S. pyogenes* FabH as would be predicted by the analysis presented here (Fig. 4). However, they are largely ineffective against *H. influenzae* FabH. When docked into the *H. influenzae* FabH structure, the benzoic acid group of the compound sterically clashes into the Phe303 (the equivalent of Phe312 in *E. faecalis* enzyme) due to its different rotamer conformation. The compounds retain some activity against *S. aureus* enzyme but weaker than expected based on the binding site similarities. This is again due to steric conflict with the equivalent Phe298 at the base of the binding site. Phe298 sidechain in *S. aureus* FabH is rotated so as to occlude the bulky benzoic acid group.

In a previous attempt to explain the substrate specificities of priming ketosynthases, which included FabH and its homolog ZhuH, it was suggested that the length of the loop L9 along with the identity of the residue at the position equivalent to Met92 of *E. faecalis* FabH might influence the shape of the acyl binding pocket thereby modulating the substrate specificity [22]. Although this is a possibility that cannot be ruled out, in light of the crystallographic data for FabH from different species with varying lengths of loops L9 and showing minimal divergence in the shapes of their acyl binding pockets, we tend to favor alternative explanation presented here. Moreover, ZhuH sequence and structure analysis and its ability to use branched chain substrate is consistent with the hypothesis proposed here (Fig. 4).

As shown here, perfect conservation of the amino acid residues that constitute the target site used for inhibitor design is not enough to assure the perfect conservation of its three-dimensional architecture. It is likely that the identities of residues that are a layer (or two) removed from the immediate vicinity of the target site might affect its shape by subtly changing the rotamer conformation of the residues lining the target site. In such a scenario, as in FabH, the daunting challenge would be to come up with a small molecule inhibitor of the enzyme that maintains the shape complementarity with the general architecture of the substrate-binding clefts of the enzymes from different species, while at the same time keeping subtle differences in active site architectures from negatively influencing the potency of the small molecule.

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References


