First X-ray Cocrystal Structure of a Bacterial FabH Condensing Enzyme and a Small Molecule Inhibitor Achieved Using Rational Design and Homology Modeling

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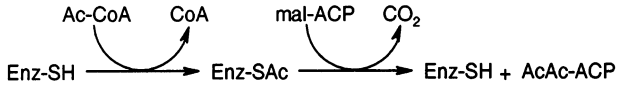
Received August 13, 2002

Abstract: The first cocrystal structure of a bacterial FabH condensing enzyme and a small molecule inhibitor is reported. The inhibitor was obtained by rational modification of a high throughput screening lead with the aid of a *S. pneumoniae* FabH homology model. This homology model was used to design analogues that would have both high affinity for the enzyme and appropriate aqueous solubility to facilitate cocrystallization studies.

β-Ketoacyl-acyl carrier protein (ACP) synthase III, also known as FabH, plays an essential role in the biosynthesis of bacterial fatty acids.[1] FabH is the initiator of the fatty acid chain elongation cycle[2] and plays a key regulatory role for the entire biosynthetic pathway via feedback inhibition by long-chain acyl ACPs.[3] FabH, a member of the family of condensing enzymes, catalyzes the cysteine-mediated Claisen condensation reaction between malonyl ACP and an enzyme-bound acetate unit derived from acetyl-CoA. The catalytic mechanism involves acetylation of cysteine by acetyl-CoA, with release of CoA, followed by condensation with the enolate derived from enzyme-mediated decarboxylation of malonyl ACP. The acetoacetyl ACP reaction product then feeds into the fatty acid biosynthesis chain elongation cycle. Whereas FabH is the initiator of the fatty acid synthesis (FAS) cycle, two additional condensing enzymes, FabB and FabF, perform the chain elongation reactions in subsequent cycles leading to long-chain acyl ACP products. While FabB and FabF are also condensing enzymes, FabH is structurally distinct.



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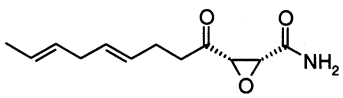
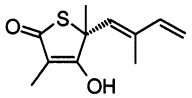
As fatty acid synthesis is an essential process for cell survival, the enzymes involved in the FAS pathway represent promising targets for broad-spectrum antibacterial agents. Cerulenin and thiolactomycin, natural-product inhibitors of the FabB and FabF condensing enzymes, both demonstrate antibacterial activity.[4] In addition, as the bacterial type II FAS is distinct from the mammalian type I FAS, there is a high probability that type II selective inhibitors can be identified.



cerulenin



thiolactomycin

A high throughput screening effort directed against *Streptococcus pneumoniae* FabH identified the indole analogue **1** (Table 1) as a potent inhibitor of the enzyme. In addition to possessing antibacterial activity against both Gram positive and Gram negative bacteria, **1** demonstrated good selectivity for the bacterial type II FAS over the human type I FAS ($IC_{50} = 30\ \mu$M, hFAS). While somewhat less potent, **1** was also shown to be a good inhibitor of *E. coli* FabH. Structure−activity studies quickly revealed the essential nature of the 2,6-dichlorobenzyl substituent. Analogues lacking this moiety demonstrated greatly reduced activity against both enzymes, indicating that the 2,6-dichlorobenzyl group played a key role in enzyme−inhibitor recognition.

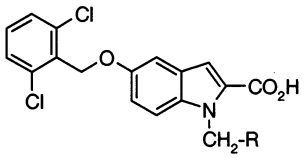
Recently, we reported X-ray crystal structures of *E. coli* FabH for both the apo-enzyme and for the acetyl-CoA complex.[5] The enzyme is characterized by having a catalytic triad, consisting of Cys112, His249, and Asn279, located at the bottom of a hydrophobic tunnel. The adenine subunit of the acetyl-CoA substrate binds to a region on the surface of the protein adjacent to the tunnel. The adenine-binding site consists principally of Trp32 and Arg151. The pantetheine of acetyl-CoA extends down the tunnel placing the acetate in proximity to Cys112. While the ACP binding site is less well defined, the pantetheine chain of malonyl ACP must also extend down the tunnel to the catalytic residues.

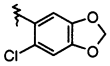
We wished to utilize this structural information to design more potent FabH inhibitors. One specific goal of our effort was to obtain X-ray crystal structures of enzyme−inhibitor complexes to support the medicinal chemistry effort. However, attempts to cocrystallize **1** with either *E. coli* FabH or *S. pneumoniae* FabH were unsuccessful, presumably a result of the very poor aqueous solubility of **1**. Docking simulations were investigated in an attempt to predict the bound conformation of **1**.[6]

Initial attempts at docking **1** into the *E. coli* FabH crystal structure were not able to produce a reliable binding model that explained key features of the available SAR. Specifically, the calculated binding modes did not account for the critical nature of the 2,6-dichlorobenzyl group. This was true when using structures derived from either the apo-enzyme or the acetyl-CoA complex. As an alternative approach, and not wanting to make significant alteration to the *E. coli* FabH crystal

10.1021/jm025571b CCC: $25.00
Published on Web 12/02/2002

Table 1. Activities vs *S. pneumoniae* and *E. coli* FabH[a,b]



compound	R	*S. pneumoniae* FabH, IC_{50} (μM)	*E. coli* FabH, IC_{50} (μM)
1		0.040	0.83
2	(4-OH)Ph	0.053	2.0
3	(4-CO_2H)Ph	0.20	2.5
4	$(CH_2)_5CO_2H$	0.17	2.0
5	$(CH_2)_4CO_2H$	0.14	7.0
6	$(CH_2)_2CO_2H$	0.062	1.7
7	CO_2H	1.2	78
8	$(CH_2)_4CONHSO_2Ph$	0.046	4.6

[a] Compounds **2**−**8** were tested as the corresponding disodium salts; compound **1** was tested as the free acid. [b] IC_{50} values are an average of two or more determinations.

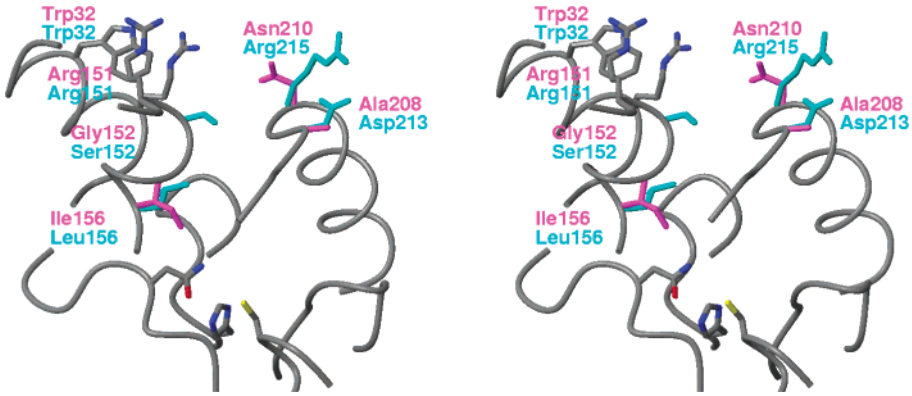


Figure 1. Overlay of active site regions of the *S. pneumoniae* FabH homology model with the *E. coli* FabH apo-enzyme crystal structure. Residues that are not common to both enzymes are highlighted in cyan (*S. pneumoniae*) and magenta (*E. coli*). The catalytic triad (Cys112, His249, Asn279, not labeled) is shown at the lower portion of the image and the adenine binding residues (Trp32, Arg151) are at the top left.

structure, a homology model of *S. pneumoniae* FabH was constructed using the reported *E. coli* FabH−CoA cocrystal as the structural template.[7] *S. pneumoniae* FabH was chosen for this work for several reasons: (1) *S. pneumoniae* FabH was the protein used for our primary assay, (2) the inhibitor demonstrated greater potency against the *S. pneumoniae* enzyme, (3) efforts were underway to crystallize the *S. pneumoniae* protein, and (4) the two enzymes are very similar within the active site region, possessing 80% identity (Figure 1).

In contrast to the *E. coli* crystal structures, docking into the *S. pneumoniae* homology model provided a rational binding mode that was consistent with the experimental data. In this predicted binding mode (Figure 2), the 2,6-dichlorobenzyl group of **1** binds within the narrow active site tunnel. While this group lies near the catalytic triad (Cys112, His249, Asn279), there is no direct interaction with these residues. The requirement of the 2,6-dichlorobenzyl group for potent activity is likely due to the exquisite shape complementarity to this region of the *S. pneumoniae* protein. Despite the conserved nature of the active sites of the *S. pneumoniae* and *E. coli* enzymes, a similar binding mode in the *E. coli* FabH crystal structure could not be achieved due to space restriction within this region.

The *S. pneumoniae* homology model also predicted that the carboxylic acid of **1** interacts with arginines on the protein surface, while the 6-chloropiperonyl group makes largely hydrophobic contacts with residues at the mouth of the active site tunnel. This placement of the 6-chloropiperonyl group locates it in close proximity to the arginine-rich region at the top of the tunnel. This suggested that replacement of this hydrophobic group with more polar side chains might be permissible. Upon the basis of these results, a series of more hydrophilic analogues were prepared that were predicted by the model to retain affinity for the enzyme while also improving compound solubility. It was believed that this approach would produce compounds suitable for cocrystallization with *E. coli* and *S. pneumoniae* FabH.

The resulting compounds **2**−**8** are shown in Table 1.[8] Incorporation of a second acidic group was chosen because of the possibility of interactions with the multiple arginine residues at the protein surface surrounding the active site tunnel. In addition, the double negative charge would increase aqueous solubility, albeit at the expense of bacterial cell penetration. With the exception of compound **7**, all analogues in Table 1 demonstrated comparable levels of inhibition to that of lead compound **1**. As predicted, these compounds lacked

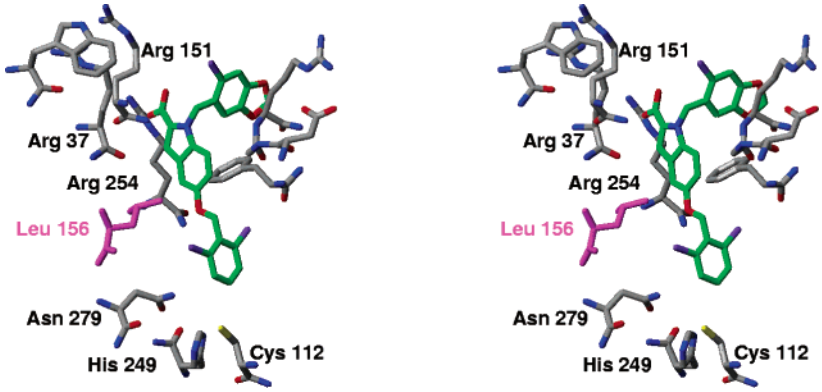


Figure 2. Predicted bound conformation of **1** in a homology model of *S. pneumoniae* FabH. Residues depicted (*S. pneumoniae* FabH numbering) correspond to those of *E. coli* FabH shown in Figure 3.

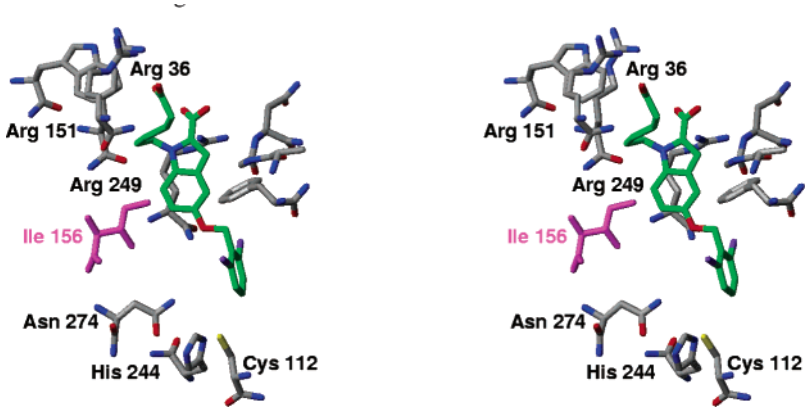


Figure 3. Cocrystal structure of compound **5** with *E. coli* FabH (*E. coli* FabH numbering).

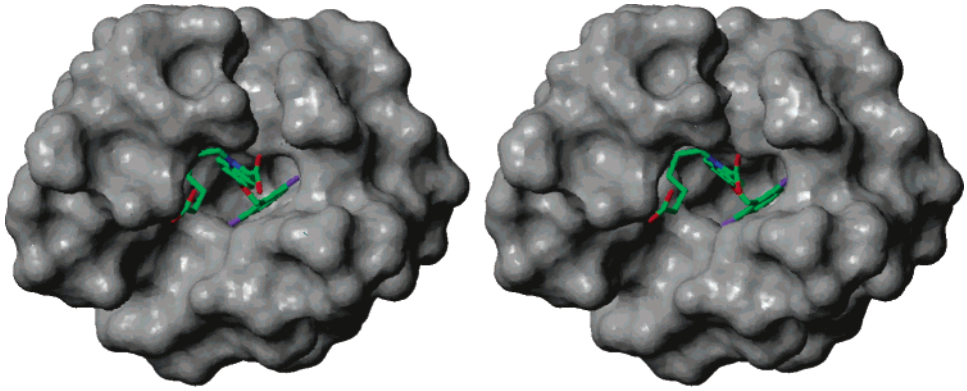


Figure 4. Surface diagram of cocrystal structure of **5** with *E. coli* FabH illustrating fit of 2,6-dichlorobenzyl group in active site tunnel. View is perpendicular to that of Figure 3 with the catalytic residues (not shown) located at the bottom of the active site tunnel.

antimicrobial activity but possessed suitable aqueous solubility to permit cocrystallization with the available FabH proteins.

Cocrystallization studies yielded an X-ray structure for the enzyme−inhibitor complex between **5** and *E. coli* FabH (Figure 3).[9] This is the first reported cocrystal structure of a FabH condensing enzyme showing the binding mode of a small molecule inhibitor. The crystallographically determined binding mode of this inhibitor is remarkably similar to that predicted by docking **1** into the *S. pneumoniae* FabH homology model. In particular, the 2,6-dichlorobenzyl group fits into the hydrophobic tunnel as predicted, with no direct interaction with the catalytic residues. One key difference is seen for Ile156 between this cocrystal structure and that of the apo-enzyme.[5] The dihedral angle X_1 is in the gauche$^+$ conformation in the apo-enzyme, and is in the gauche$^-$ conformation in the cocrystal structure (compare with Figure 1). Along with a small shift in the backbone angles, this change in dihedral angle opens enough space in the binding site pocket to accommodate the second chlorine of the 2,6-dichlorobenzyl group. As noted, this added space was lacking in the previous *E. coli* FabH crystal structures and, therefore, prevented the successful docking of **1**.

Additional key interactions observed in the X-ray structure include a water-mediated interaction between the C-2 carboxylic acid and Arg36 as well as a salt bridge between the hexanoate side chain and Arg151, a residue that forms part of the adenine binding site of acetyl-CoA.[5] The structure further reveals that compound **5** binds to the apo-form of the enzyme (Cys112

is not acetylated) as predicted by kinetic studies (data not shown). With the 2,6-dichlorobenzyl group filling the active site tunnel, the acetyl-CoA substrate is prevented access to the catalytic residues and is blocked from acetylating Cys112, the first step in the catalytic cycle.

In comparing the predicted binding mode of **1** (Figure 2) with the actual bound structure of **5** (Figure 3), it appears that important elements for recognition exist. First, the 2,6-dichlorobenzyl group is required to fill a complementary hydrophobic region within the active site tunnel (Figure 4). Second, the presence of an acidic group is needed in order to form an ionic interaction with one of the arginine residues at the top of the active site tunnel (either directly or through water). For the newly synthesized analogues, an additional binding element has been incorporated via a second acidic group. The data in Table 1 suggests that the acceptable chain length for this second acidic group is four to seven carbon units with four appearing optimal. Compound **7**, containing the acetate side chain, is likely to be too short to make optimum contacts with one of these arginine residues. The indole itself appears to merely serve as a scaffold on which these three binding elements are arranged. Interestingly, the crystal structure does not shed additional light on the nature of the 6-chloropiperonyl binding of **1**.

The present study describes the use of a *S. pneumoniae* FabH homology model to design inhibitors with favorable physical properties to facilitate cocrystallization studies. While lead compound **1** contained a hydrophobic group at the 1-position of the indole, docking calculations suggested that more polar groups would be tolerated within this region. The resulting structural modifications led to potent inhibitors with improved aqueous solubility. This approach has resulted in the first X-ray crystal structure of an enzyme-small molecule inhibitor complex for a FabH condensing enzyme. The information obtained from this cocrystal will be used for the rational design of more potent inhibitors of this novel and unexploited antibacterial target.[10] Of significance is the ability to use a FabH from one bacteria, in this case *S. pneumoniae*, to design inhibitors of FabH from multiple bacteria, i.e., *S. pneumoniae* and *E. coli*. This will be a critical element in obtaining broad-spectrum antibacterial agents that inhibit FabH from many different organisms.

The atomic coordinates of the cocrystal structure of **5** with *E. coli* FabH have been deposited in the RCSB Protein Data Bank with accession code 1MZS.

Supporting Information Available: Experimental details and structural characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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(6) All docking calculations were performed using the program Flo; see McMartin, C.; Bohacek, R. S. QXP: powerful, rapid computer algorithms for structure-based design. *J. Comput.-Aided Mol. Design* **1997**, *11*, 333−344. No structural waters were included in docking calculations in *S. pneumoniae* FabH.

(7) The model was built using the MOE modeling package, Chemical Computing Group, Montreal Canada.

(8) For enzyme assay conditions see Khandekar, S. S.; Gentry, D. R.; Van Aller, G. S.; Warren, P.; Xiang, H.; Silverman, C.; Doyle, M. L.; Chambers, P. A.; Konstantinidis, A. K.; Brandt, M.; Daines, R. A.; Lonsdale, J. T. Identification, Substrate Specificity, and Inhibition of the *Streptococcus pneumoniae* β-Ketoacyl-Acyl Carrier Protein Synthase III (FabH). *J. Biol. Chem.* **2001**, *276*, 30024−30030.

(9) Attempts at crystallizing *S. pneumoniae* FabH with and without inhibitors have been unsuccessful.

(10) For two recently reported inhibitors of *S. aureus* FabH see Xin, H.; Reynolds, K. A. Purification, Characterization, and Identification of Novel Inhibitors of the β-Ketoacyl-Acyl Carrier Protein Synthase III (FabH) from *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **2002**, *46*, 1310−1318.

JM025571B