FabGs, or $\beta$-oxoacyl reductases, are involved in fatty acid synthesis. The reaction entails NADPH/NADH-mediated conversion of $\beta$-oxoacyl-ACP (acyl-carrier protein) into $\beta$-hydroxyacyl-ACP. HMwFabGs (high-molecular-weight FabG) form a phylogenetically separate group of FabG enzymes. FabG4, an HMwFabG from Mycobacterium tuberculosis, contains two distinct domains, an N-terminal ‘flavodoxin-type’ domain and a C-terminal oxoreductase domain. The catalytically active C-terminal domain utilizes NADH to reduce $\beta$-oxoacyl-CoA to $\beta$-hydroxyacyl-CoA. In the present study the crystal structures of the FabG4–NAD binary complex and the FabG4–NAD$^+$–hexanoyl-CoA ternary complex have been determined to understand the substrate specificity and catalytic mechanism of FabG4. This is the first report to demonstrate how FabG4 interacts with its coenzyme NADH and hexanoyl-CoA that mimics an elongating fattyacyl chain covalently linked with CoA. Structural analysis shows that the binding of hexanoyl-CoA within the active site cavity of FabG significantly differs from that of the Ci$\alpha$ fattyacyl substrate bound to mycobacterial FabI [InhA (enoyl-ACP reductase)]. The ternary complex reveals that both loop I and loop II interact with the phosphopantetheine moiety of CoA or ACP to align the covalently linked fattyacyl substrate near the active site. Structural data ACP inhibition studies indicate that FabG4 can accept both CoA- and ACP-based fattyacyl substrates. We have also shown that in the FabG4 dimer Arg$^{146}$ and Arg$^{445}$ of one monomer interact with the C-terminus of the second monomer to play pivotal role in substrate association and catalysis.

Key words: co-operativity, hexanoyl-CoA, Mycobacterium tuberculosis, short-chain dehydrogenase/reductase, $\beta$-oxoacyl reductase.

INTRODUCTION

Type II fatty acid metabolism in Mycobacterium tuberculosis is particularly important for the synthesis of very-long-chain fatty acids [1]. Very-long-chain fatty acids are further processed to yield a meromycolate backbone and are ultimately destined for mycolic acid synthesis. The enzyme FabG is committed to the second step of type II fatty acid synthesis where NADPH is used to reduce $\beta$-oxoacyl-CoA to $\beta$-hydroxyacyl-CoA. The M. tuberculosis genome consists of multiple FabG genes [2], two out of five of which are conserved among mycobacterial species. FabG1 (MabA) is associated with type II fatty acid metabolism [3], whereas FabG4 in mycobacteria belongs to an operon possibly involved in a non-conventional processing of fatty acids [4,5]. McFadden and co-workers have shown that FabG4 is essential for mycobacterial growth in Roisin's minimal medium [6]. Functional complementation in yeast reveals that FabG4 can participate in the fatty acid biosynthetic pathway [7]. FabG4 is expressed in Sauton’s medium with a sub-inhibitory concentration of streptomycin [8] and hence it may play a role in drug resistance in mycobacterial species.

HMwFabG (high-molecular-weight FabG) is a distinct group of $\beta$-oxoacyl reductases mostly found in bacteria including Actinobacteria and certain lipid rich Proteobacteria. Mycobacterial FabG4 belongs to the HMwFabG group. FabG1, in contrast, belongs to the LMwFabG (low-molecular-mass FabG) group. There are two apparent differences between these two groups. First, HMwFabGs possess an N-terminal domain containing a flavodoxin-type fold. This N-terminal domain is absent in LMwFabGs. In HMwFabGs the domain is primarily responsible for stable oligomeric assembly [9]. Secondly, the C-terminal catalytic domain of HMwFabG requires NADH as hydride donor. The C-terminal domain is responsible for $\beta$-oxoacyl reductase activity which is common to both HMwFabGs and LMwFabGs, such as EcFabG (Escherichia coli FabG) and MiFabG1 (M. tuberculosis FabG1), but the LMwFabGs require NADPH for catalysis. Although FabG4 requires NADH for its $\beta$-oxoacyl-CoA reductase activity [7,9], it does not share any structural similarity or sequence homology with the NAD$^+$-utilizing 3-hydroxyacyl-CoA dehydrogenase that is required for the $\beta$-oxidation pathway [10].

FabG is homologous to the enoyl reductase (FabI) and the only ternary complex structure of a reductase of fatty acid metabolic pathway is mycobacterial FabI [InhA (enoyl-ACP reductase)] complexed with NAD$^+$ and C16 fattyacyl N-acetyltyasmine substrates (PDB code 1BVR [11]). Although FabG and FabI are structurally homologous they are distinguishable in several aspects like sequence similarity, substrate specificity, active-site architecture and length of the $\alpha$-helix in their subdomain [3,12,13]. Both FabG and FabI accept fattyacyl substrates covalently linked with either CoA or the phosphopantetheine moiety of ACP. Owing to the lack of a whole phosphopantetheine moiety in the Ci$\alpha$ fattyacyl substrate of the InhA–substrate complex structure, the interaction
between the entire phosphopantetheine part and FabG cannot be proposed from the homologous InhA and its substrate-bound structure. Moreover, the structure of EcFabI complexed with ACP (PDB code 2FHS) accompanied by a molecular dynamics simulation indicates that the interaction between EcFabI and acylpantetheine phosphate is mediated through a protruding α-helical subdomain and catalytic loop II [14]. NMR studies have revealed that in EcFabG ACP sits on Arg128 of the α4 helix and Arg172 of the α5 helix, which are not part of the α-helical subdomain and loop II [15]. It follows that FabG–ACP interaction differs significantly from FabI–ACP interaction. FabG adheres to the phosphopentethine moiety of the ACP and brings the β-oxo group of the substrate near the active site. Since the same phosphopantetheine moiety is also present in CoA, it must communicate with FabG in a similar fashion. To date there is no report about FabG and CoA interaction. The C-terminal domain of FabG4 (residues 215–454) shares a 32–41% sequence homology with LMwFabGs from various organisms [16]. Additionally the Cα r.m.s.d. (root mean square deviation) between the C-terminal domain of FabG4 and LMwFabGs varies from 0.655 Å (1 Å = 0.1 nm) to 1.051 Å. Hence FabG4 interaction with a CoA-linked substrate can be thought of as an LMwFabG and CoA substrate interaction. In the present study we have attempted to characterize an HMwFabG for the first time in terms of its coenzyme and substrate specificity. This is the first report of a FabG4–NAD⁺–hexanoyl-CoA complex structure showing how the CoA-linked substrate mimetic interacts with a β-oxoacyl reductase. Structural data and ACP inhibition studies revealed that FabG4 is a NADH-dependent β-oxoacyl reductase and can accept the β-oxo fattyacyl group covalently linked with CoA or ACP for catalysis. Additionally, the ternary complex provides the first structural proof of the FabG catalytic mechanism. Mutational analysis supporting the structural data confirmed that the C-terminus of FabG4, together with Arg446 and Arg450, plays a crucial role in catalysis.

**EXPERIMENTAL**

**Materials**

Plasmid pQE30, *E. coli* M15 or SG13009 cells were purchased from Qiagen. The enzymes used in the molecular biology studies were from Fermentas. Acetoacetyl-CoA, hexanoyl-CoA, DL-hydroxybutyric acid, DL-hydroxybutyryl-CoA, NAD⁺ and NADH were from Sigma Chemicals.

**Cloning and site-directed mutagenesis**

Cloning of wild-type FabG17–454 was as described previously [9]. Truncation of the first 16 amino acids did not affect the activity of the protein. The sequences of the primers used in PCR are listed in Supplementary Table S1 (at http://www.biochemj.org/bj/450/bj4500127add.htm). The single mutants R146A and R445A and the double mutant R146A + R445A were generated with the double overlap extension method [17] using Pfu DNA polymerase. DNA sequencing was performed to confirm the mutations. Amplicons and the pQE30 vector were digested with the corresponding restriction enzyme pairs (either BamHI/HindIII or SacI/HindIII). The digested PCR products were ligated with the pQE30 vector using T4 DNA ligase. The pQE30 vector anchoring the mutants was transformed into chemically competent *E. coli* M15 or SG13009 cells. Positive clones were selected against a double antibiotic (ampicillin and kanamycin)-containing LB (Luria–Bertani) agar plate.

**Overexpression, purification and crystallization**

Overexpression and purification of FabG17–454 were performed as described previously [9]. Two single mutants and one double mutant were overexpressed and purified using the same protocol. Purified FabG4 was concentrated to 20 mg/ml using a 10 kDa-cutoff Vivaspin 20 concentrator (GE Healthcare). The protocol used to obtain the NADH-bound crystals was described previously [16]. To obtain the hexanoyl-CoA and NAD⁺ ternary complex, FabG4 was concentrated and mixed in NAD⁺ at a 1:5 molar ratio. Hexanoyl-CoA trilithium salt was simultaneously dissolved in crystallization solution containing 45% (v/v) polypropylene glycol P400 and 0.1 M Mes (pH 6.5) to make a 2 mM stock. Crystals of the ternary complex were produced by hanging-drop vapour diffusion by mixing 2 μl of the protein/NAD⁺ mixture with 2 μl of crystallization condition containing 2 mM hexanoyl-CoA. The R146A + R445A double mutant of FabG4 was incubated with NADH at a 1:5 molar ratio and subjected to crystallization trials. Despite several efforts no crystals were obtained for this mutant.

**Data collection and structure determination**

All of the diffraction data were collected in-house with a Rigaku Micromax HF007 Microfocus CuKα (1.54 Å) rotating anode generator and Rigaku Raxis IV++ detector system. Prior to mounting, crystals were cryoprotected with crystallization condition and flash cooled with a liquid nitrogen stream. The data collection protocol for the FabG4–NADH complex was described previously [16]. For the FabG4–NAD⁺–hexanoyl-CoA ternary complex the data were collected for 180° with a 1° oscillation per frame and crystal-to-detector distance of 120 mm. The data collection statistics are shown in Table 1. All diffraction data were processed using XDS [18] and scaled with SCALA [19] from the CCP4 interface [20]. The structure of the FabG4–NADH complex was determined by molecular replacement in MOLREP [21] using a monomer of the apoFabG4 structure (PDB code 3LLS). The initial phase of the acetoacetyl-CoA-soaked structure and ternary complex structure were obtained using the monomer of the apoFabG4 structure (PDB code 3LLS) as a model. The initial solutions were subjected to refinement using Refmac5 [22]. Several cycles of restrained refinement followed by NCS refinement were used where necessary. The structures were built in Coot [23] and refined with a few cycles of TLS (Translation–Libration–Screw) refinement to improve the quality of the model. The co-ordinates of the ligands NADH and hexanoyl-CoA were obtained from PRODRG [24]. Ligand fitting was done either manually or by using the Phenix Ligfit module [25]. Proper stereochemistry of the ligand was chosen according to the difference density map generated from the ligand-free model. All the atoms of hexanoyl-CoA can be traced in electron density except the 3’ ribose phosphate. The stereochemistry of the structures was checked using the RAMPAGE server [26] and SFCHECK from the CCP4 interface [20].

The structure factor files and co-ordinates of the binary and ternary complexes are deposited in the PDB under the accession codes 4FW8 and 3V1U. Final refinement statistics are summarized in Table 1. The figures were prepared with PyMOL (http://www.pymol.org) visualization software. The structure–sequence alignment was done using ESPript [28].

**Kinetic analysis and enzymatic activity**

Kinetic analysis of FabG4 and its mutants were performed using the substrates acetoacetyl-CoA and NADH. Rate measurement was carried out at 340 nm at 25 °C in an Evolution™
I (hkl) is the average intensity. R_{ave} = \frac{\sum_{hkl} |F_{obs}| - |F_{calc}|/ \sum_{hkl} |F_{obs}| \times 100\%}{\text{where } F_{obs} \text{ and } F_{calc} \text{ are observed and calculated structure factors. R_{ave} is calculated for a random set of 5\% of reflections not used in the refinement. ZPG, polypropylene glycol P460.}

Table 1 Data collection and refinement parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>FabG4–NADH</th>
<th>FabG4–NAD+–hexanoyl-CoA</th>
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<td>PDB code</td>
<td>4FW8</td>
<td>3V1U</td>
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<td>Data collection statistics</td>
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<td>Resolution range (Å)</td>
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<td>a = b = 102.05, c = 76.05</td>
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<td>122189 (17298)</td>
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<td>Number of unique reflections</td>
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<td>16296 (2309)</td>
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<td>Allowed region (%)</td>
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<td>Outlier region (%)</td>
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Table 2 Kinetic parameters of wild-type FabG4 and its different mutants

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<th>Ligand Parameter</th>
<th>Parameter</th>
<th>Wild-type</th>
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<th>R445A</th>
<th>R146A + R445A</th>
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</thead>
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<td>NADH</td>
<td>k_{cat} (s⁻¹)</td>
<td>318.90 ± 16.75</td>
<td>146.42 ± 4.47</td>
<td>28.78 ± 0.89</td>
<td>13.23 ± 1.06</td>
</tr>
<tr>
<td></td>
<td>K' (µM)</td>
<td>24.15 ± 2.56</td>
<td>25.01 ± 1.5</td>
<td>27.88 ± 1.61</td>
<td>26.78 ± 3.54</td>
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<td></td>
<td>k_{cat}/K' (s⁻¹·µM⁻¹)</td>
<td>13.15</td>
<td>5.85</td>
<td>1.03</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>σ</td>
<td>1.64</td>
<td>1.93</td>
<td>2.07</td>
<td>1.67</td>
</tr>
<tr>
<td>Acetoacetyl-CoA</td>
<td>k_{cat} (s⁻¹)</td>
<td>453.96 ± 8.05</td>
<td>350.12 ± 9.83</td>
<td>29.80 ± 1.34</td>
<td>28.31 ± 1.44</td>
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<tr>
<td></td>
<td>K' (µM)</td>
<td>141.94 ± 4.72</td>
<td>145.42 ± 7.45</td>
<td>229.76 ± 20.89</td>
<td>248.44 ± 23.33</td>
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<td>k_{cat}/K' (s⁻¹·µM⁻¹)</td>
<td>3.19</td>
<td>2.40</td>
<td>0.13</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>σ</td>
<td>1.83</td>
<td>2.06</td>
<td>1.24</td>
<td>1.28</td>
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</tbody>
</table>

300 UV–visible spectrophotometer (Thermo Fisher Scientific) in a buffer containing 50 mM HEPES (pH 7.4). The total reaction volume was kept to 500 µl with a final protein concentration of 1 µM. Reductase activity was measured by monitoring the decrease in absorbance following NADH into NAD⁺ conversion. The apparent K' values for both NADH and acetoacetyl-CoA were determined by varying one substrate concentration while maintaining another substrate concentration at a fixed saturation level. Acetoacetyl-CoA concentration was varied from 0 to 1.0 mM keeping the NADH concentration at 0.3 mM. NADH concentration was varied from 0 to 0.15 mM keeping the acetoacetyl-CoA concentration at 0.6 mM. To measure the dehydrogenase activity NAD⁺ and β-hydroxybutyryl-CoA were used. NAD⁺ concentration was kept at fixed saturation level of 0.6 mM and β-hydroxybutyryl-CoA or β-hydroxybutyric acid was varied from 0 to 2.5 mM. Similarly, to determine the kinetic parameters for NAD⁺, β-hydroxybutyryl-CoA concentration was kept fixed at 2.0 mM with varying concentration of NAD⁺ from 0 to 0.6. The data were fitted to the equation \( y = \frac{V_{max} [S]}{K_S + [S]} \), where y denotes the change in D_{abs} and [S] denotes substrate concentration. K is a constant comprising of interaction factors and dissociation constant. The kinetic data are summarized in Table 2.
Computational analysis and protein–protein docking analysis

Protein–protein docking was used to understand the possible mode of ACP interaction with FabG. *M. tuberculosis* ACP (AcpM/Rv2244; PDB code 1KLF) was chosen as ligand. *MtbFabG1* (MabA; PDB code 1UZM) and FabG4 (PDB codes 3L5S and 3Q6I) were chosen as the receptor. Protein–protein docking was performed using different receptor and ligand combination in the GRAMMJX [29] and ZDOCK [30] servers. CaspP server [31] was used to calculate the surface area and volume of the catalytic groove. PDB-PISA server was used to calculate the different parameters related interface [32].

Cloning, overexpression and purification of AcpM

The cloning overexpression and purification of mycobacterial AcpM (Rv2244) are detailed in the Supplementary Online Data (at http://www.biochemj.org/bj/450/bj4500127add.htm). In brief, AcpM was amplified from the *M. tuberculosis* H37Rv genome and the amplified product was ligated with the pQE30 vector to clone into chemically competent E. coli M15 cells. Positive clones are selected by ampicillin and kanamycin-containing LB media. E. coli M15 cells containing the acpM-anchored pQE30 vector were grown until the *D*$_{600}$ reached 0.6 in 2 litres of LB medium containing antibiotics and induced with IPTG (isopropyl-β-D-thiogalactopyranoside). The induced cells were allowed to grow for 4 h after which they were harvested and lysed with sonication. Sonication was performed using a Microson™ ultrasonic cell disruptor (Misonix) using a 30 s pulse at regular intervals of 60 s. The entire procedure was repeated twice to ensure complete lysis. The supernatant was collected to load on to a Ni-NTA (Ni$^{2+}$-nitrilotriacetate) column. AcpM purified using Ni-NTA chromatography was subjected to buffer exchange and further purified using Q Sepherose anion-exchange chromatography. The final step of purification was done using S75 gel-filtration chromatography.

ACP-inhibition assay

Purified AcpM was subjected to extensive dialysis against assay buffer containing 50 mM Heps (pH 7.4). Reactions were carried out in assay buffer containing 1 µM of FabG4 and 0.3 mM NADH. Rate measurement was carried out as described above. To determine the dose–response curve different AcpM concentrations were used (5–40 µM) in presence of 1 mM acetocetyl-CoA. A dose–response curve was obtained by plotting the activity of FabG4 against log[AcpM]. The data were fitted to the equation $y = \text{Min} + \frac{(\text{Max} - \text{Min})}{1 + \left(\frac{[AcpM]}{K_{IC50}}\right)^n}$, where Min and Max are lower and higher asymptotes of the curve. Activity is denoted by $y$ and $x$ denotes the concentration of AcpM. The IC$_{50}$ value was calculated from the curve at which concentration of AcpM 50% of FabG4 activity is retained compared with the initial value and $n$ indicates the Hill coefficient. For the activity profile acetocetyl-CoA concentration was varied from 50 µM to 1 mM at several fixed concentrations of AcpM.

RESULTS AND DISCUSSION

Overall structure of the FabG4–NADH complex

The crystal structure of *M. tuberculosis* FabG4 complexed with NADH was determined to a 2.79 Å resolution. These crystals belonged to the P1 space group with two dimers in a unit cell. The overall topology and the core structure of FabG4–NADH binary complex is very similar to the apoFabG4 structures (PDB codes 3LLS and 3M1L) [9]. The FabG4–NADH binary structure is comprised of two distinct domains and one subdomain. The N-terminal domain (residues 1–196) is folded into a flavodoxin-type fold and the C-terminal domain (residues 215–454) is a typical β-α-β Rossman fold commonly found in short-chain dehydrogenase/reductase family proteins [33] (Figure 1A). Briefly, the N-terminal domain consists of centrally placed β-strands, β1A–β3A–β4A–β6A–β7A, flanked by α1A, α3A, α5A and α6A helices. The C-terminal domain consists of β-strands β1B–β2B–β7B–β5B–β1B–β2B–β4B surrounded by α-helices α1B, α3B, α4B, α6B, α8B and α9B. The loop regions between β5B–α6B and β7B–α8B are known as loop I and loop II respectively. The α’ subdomain is a protruding part of the region between β9B and α9B housing the α’-helix. Asn193, Ser237, Tyr250 and Lys264 constitute the active site of FabG4. Dimeric interfaces are formed by extensive hydrophobic contacts between α3A with α6B’ and α5A with α8B’. The dimer–dimer interface is formed via minimal residue contacts between α3B of one monomer and the loop joining β7A–β1B of another monomer.

The FabG4–NADH structure shows that NADH is bound in extended syn conformation (Figure 1B). NADH is accommodated within the dinucleotide-binding crevice created by the C-terminal domain. The amide group of the adenosine moiety of NADH takes part in hydrogen bonding with Asp254 (Figure 1B). Superimposition of NADP$^+$-bound LMwFabG [EcFabG, MtbFabG1, BnFabG (Brassica napus FabG) and SaFabG1 (Staphylococcus aureus FabG1)] structures (Supplementary Figure S1 at http://www.biochemj.org/bj/450/bj4500127add.htm) onto the NADH-bound FabG4 structure reveals that the corresponding adenosine 2 phosphate accommodating region in FabG4 is less voluminous and comprised of a rigid secondary structure. The amino acid sequence of the 2-phosphate-accommodating region of FabG4 is D130VESA98 (Figure 2). Asp264, which is conserved in HMwFabGs, juts out from the C-terminus of the β2B strand to make hydrogen bonds with the 2’ and 3’ hydroxyl groups of the adenosine ribose (Figure 1B). Structural superimposition also shows that the amino acid type and its orientation at the corresponding position of Asp237 are important for discrimination between NADH and NADPH as it determines the volume of the 2-phosphate-accommodating region. Analysis of the FabG4–NADH complex with other LMwFabG–NADP complexes confirms the role of an arginine residue in two different conformations of pyrophosphate moiety of NAD(P)$^+$. In one conformation both the phosphate moieties reside nearest to the β1B–α1B loop and in an alternative conformation the phosphodiester bond is rotated to allow only one phosphate nearest to the β1B–α1B loop. In the FabG4–NADH complex Arg237 directs the pyrophosphate in the second conformation (Figure 1B). Nicotinamide ribose is involved in hydrogen bonding interaction with active site Tyr360 and Lys364 of the α8B helix, as found in LMwFabGs [34]. The active-site residue Ser417 is located in loop II, whereas Asn319 is a part of the α6B helix around which this helix forms a kink to maintain a certain distance from the catalytic site. This is particularly important because it participates in a proton circuit that is essential for catalytic mechanism [34]. The protein–NADH interaction data are summarized in Supplementary Table S2 (at http://www.biochemj.org/bj/450/bj4500127add.htm).

Structural comparison reveals two striking differences between the NADH–FabG4 complex and the apoFabG4 structure. First, in the FabG4–NADH complex eight residues (residues 20–28) of the N-terminal domain can be traced in electron density. Residues 21–26 form a short α-helix (α$_2$) in chain B only. The stability of this region is attributed to the crystal contacts. Secondly, residues 396–409 of the α’ subdomain are traced and the subdomain
Figure 1  NADH interaction with FabG4

(A) Cartoon representation of the NADH-bound FabG4 structure. Broken lines are to show the connectivity of the structure. The N-terminal residues (20–28) are found in helical conformation ($\alpha_n$). Subdomain $\alpha'$ is made of a helix located away from the core structure. The loop joining $\beta_{5B}$–$\alpha_{5B}$ is known as loop I and the $\beta_{7B}$–$\alpha_{8B}$ loop housing $\alpha_{7B}$ is known as loop II. Arg146 and Arg445 are shown. (B) Stereo view of $F_o$–$F_c$ difference electron density map contoured at 3.0 $\sigma$ showing the fitting of NADH. Residues either interacting with or located in close proximity to the NADH are shown. Asn319, Ser347, Tyr360 and Lys364 constitute the active-site residues. is found in an open conformation. Thr$^{395}$ and Ile$^{395}$ of the $\alpha'$ subdomain make hydrogen bonds with the amide group of the nicotinamide ring. The subdomain forms a lid-like structure which is common among short-chain dehydrogenase/reductase members [11]. The subdomain regulates the accessibility of the active site and creates a solvent-free environment during catalysis [11]. Two proline residues, Pro$^{193}$ and Pro$^{222}$, have been identified near the hinge of this lid among polyketide oxoreductases [35]. The corresponding proline residues in FabG4 or any HMwFabG are absent, but two signature motifs, A$^{389}$PG$^{391}$ and Q$^{417}$GG$^{419}$, were identified at the N- and C-terminus of this lid (Figure 2). The presence of Gly$^{391}$ and Gly$^{418}$ accounted for the lid’s flexibility. Pro$^{402}$ in the $\alpha'$ subdomain is conserved among HMwFabGs and renders a rigid relative conformational orientation to the $\alpha'$-helical subdomain.

Overall structure of the FabG4–NAD$^+$–hexanoyl-CoA ternary complex

Crystals of the FabG4–NAD$^+$–hexanoyl-CoA ternary complex belong to the space group $P3_21$ with a single monomer in the asymmetric unit. The overall structure of the ternary complex is the same as the FabG4–NADH binary complex. NAD$^+$ sits on the dinucleotide binding $\beta$-$\alpha$-$\beta$ motif of FabG4. Hexanoyl-CoA sits almost perpendicularly to the NAD$^+$-binding crevice (Figure 3). All the atoms of the hexanoyl-CoA can be traced in electron density map except the 3' adenosine ribose phosphate.

The ribose sugar of hexanoyl-CoA adopts a 3'-endo puckered conformation with syn-glycosidic rotation angle and binds with the protein (Figure 3). The adenosine moiety of hexanoyl-CoA is bent approximately 74$^\circ$ around the ribose part and partly
Figure 2  Multiple sequence alignment of different HMwFabGs

Active-site residues are denoted by an asterisk, grey boxes indicate NADH-interacting residues and grey circles indicate hexanoyl-CoA-binding residues. Sequence rigidity between $\beta_{2B}$ and $\alpha_{3B}$ is responsible for NADPH rejection. The loop I and II spans are highlighted. Distribution of hydrophobic residues A296GIT299 of loop I and 315, 318 of $\alpha_{6B}$ are conserved presumably responsible for harbouring the hydrophobic fattyacyl chain. The locations of Arg146 and Arg445 are denoted by arrowheads. Two regions highlighted by boxes G391FI393 and Q407GG409 denote the boundary regions of $\alpha'$ subdomain. Pro402 is possibly responsible for a rigid relative conformation of the two regions of the $\alpha'$ subdomain. Pro445 is possibly responsible for a rigid relative conformation of the two regions of the $\alpha'$ subdomain. The C-terminal residues are populated with hydrophobic residues and are important for efficient catalysis. TT represents strict $\beta$-turns. Mtb, M. tuberculosis; Mlp, M. leprae; Cul, Corynebacterium urealyticum; Nfa, Nocardia farcinica; Roso, Ralstonia solanacearum; Sco, Streptomyces coelicolor; Psy, Pseudomonas syringae.

interacts with the dimeric interface. The bend in CoA is also found in other CoA-bound dehydrogenase structures [36,37]. At the dimeric interface Arg153/B and Glu151/B of the second monomer interact with hexanoyl-CoA. Arg153/B resides at the middle of the $\alpha$5A and $\beta$6A loop and is involved in hydrogen bonding with adenosine N7. Structural comparison between the binary and ternary complexes shows that Arg153/B exerts a conformational rearrangement to allow hexanoyl-CoA to dock on the FabG4. Arg153/B is not conserved among HMwFabGs and therefore is not essential for substrate positioning. However, Glu151/B is a conserved residue. Glu151/B interacts with the amide group of the adenosine ring via a backbone carbonyl group. Three residues, Lys302, Leu303 and Asn306, of loop I interact with the adenosine 5' diphasphate group of hexanoyl-CoA. Lys302 and Asn306 interact with hexanoyl-CoA via side chains, whereas Leu303 is involved in interaction via a backbone peptide nitrogen. A multiple sequence alignment of different HMwFabGs shows that the position of Lys302 and Asn306 are always occupied by polar residues (Figure 2). The pantetheine portion of the hexanoyl-CoA makes a hydrogen bond with the conserved Asp301 and Asn354. Asp301 is located in loop I and Asn354 in loop II. Glu357 and Ser347 form a hydrogen-bonding network with water molecules near the active site to align the substrate thioester bond which consequently disposes the $\beta$-carbon of the fattyacyl chain near to the active site. The hydrophobic fattyacyl chain is then bent towards the hydrophobic passage created by the loop I and $\alpha$6B to house the elongated fattyacyl chain. The protein–hexanoyl-CoA interactions are listed in Supplementary Table S3 (at http://www.biochemj.org/bj/450/bj4500127add.htm).

Comparison of the binary and ternary complexes does not show any significant difference; however, there are some local rearrangements of the residues surrounding the catalytic site and the NAD$^+$-binding site. The pyrophosphate portion of NAD$^+$ in the ternary complex sits differently than that of NADH. Their conformations are guided by the Arg223 as discussed above. Although there is a difference in planarity in the nicotinamide ring in NAD$^+$ and NADH, in the ternary complex the nicotinamide moiety is $13^\circ$ more inclined towards the active site. The $\alpha'$ subdomain is also present in ternary complex structure without any considerable crystal contacts.

The substrate-binding site of FabG is different from FabI

In the FAS (fatty-acid synthase)-II pathway of fatty acid metabolism there are two reductases involved, FabG and FabI, and the only substrate-bound structure available currently is for FabI. The ternary complex of mycobacterial FabI (InhA)–NAD$^+$–C16 fattyacyl N-acetylcysteamine (PDB code 1BVR) provides the
Structure–function relationship of HMwFabG

Figure 3  The ternary complex of FabG4, NAD and hexanoyl-CoA

(a) FabG4 dimer along the dimeric interface. Hexanoyl-CoA (HXC) sits along the length of loop I. The adenosine part is accommodated near the dimeric interface. The α′ helical subdomain adopts an open conformation. The region enclosed by the box is shown in the close up view of hexanoyl-CoA and FabG4 interaction (b). Glu151 and Arg153 of the second monomer interacts with adenosine part of hexanoyl-CoA. Both loop I and loop II participated in hexanoyl-CoA interaction and help orientate the β-oxo group towards the active site. Catalytic Ser347 is found to interact with thioester carbonyl oxygen. (c) FabG4 dimer along the domain interface. The region enclosed by the box shows the hydrophobic hexanoyl part bends towards the hydrophobic patch of loop-I and α6B (d). Hydrophobic amino acid positions are conserved among the HMwFabGs.

first structural insight into the FabI substrate interaction [11]. The C_{16} fattyacyl substrate is a synthetic fattyacyl substrate mimetic of FabI with a trans double bond at 2-carbon position (Figure 4). However, the substrate mimetic lacks the 4′ phosphopantetheine moiety that is present in either ACP or CoA acting as a substrate holder (Figure 4). Because FabG and FabI are structurally similar and both the enzymes participate in the FAS-II pathway, the FabG4–NAD^+–hexanoyl-CoA ternary complex (PDB code 3V1U) was compared with the MtfabI–NAD^+–C_{16}-fattyacyl substrate ternary complex (PDB code 1BVR). Like C_{16} fattyacyl substrate, hexanoyl-CoA is used as a substrate (acetoacetyl-CoA) mimic for FabG, but the underlying differences are that hexanoyl-CoA has the 4′ phosphopantetheine which is common in either ACP or CoA and the fattyacyl chain is fully saturated (Figure 4). Both FabG4 and FabG must interact with the substrates in a similar manner because these proteins share considerable structural (the r.m.s.d. between the C-terminal domain of each protein from different sources ranges from 0.6 to 1.3 Å) and sequence (sequence identity ranges from 32 to 41%) homology [16].

In substrate- and cofactor-bound structure of MtfabI or InhA, Rozwarski et al. [11] defined two portals for substrate accessibility: the major and minor portals. The major portal defines the way to access the active site parallel to the NADH-binding region. The major portal is wide open and the substrate has to access the α′-helical subdomain to reach the active site. In contrast, the minor portal defines the way along the length of loop I. The accessibility is narrower and the substrate has to interact with loop I and the dimer-forming α4- and α5-helices. The major and minor portals of FabG4 are shown in Figure 5. The active form of FabG4 is a dimer. The surface potential map shows that the dimeric interface is also involved in hexanoyl-CoA association where a positively charge cluster (Figure 5) interacts with the diphosphate moiety of hexanoyl-CoA.
Figure 4 Different substrates and substrate mimics of FabG and FabI

I, β-oxobutyryl-ACP, β-Oxobutyric acid covalently linked with holoACP. II, β-oxobutyryl-CoA, β-Oxobutyric acid covalently linked with CoA. III, hexanoyl-CoA substrate mimic. The β-oxo fatty acid part is substituted with hexanoic acid. IV, C₁₆ fattyacyl N-acetylcysteamine substrate mimic of FabI used in [11]. The long hexadecanoic acid is covalently linked with N-acetylcysteamine which partly mimics the 4′ phosphopantetheine moiety of either ACP or CoA.

Figure 5 The major and minor portals of FabG4 dimer:

(a) The major portal is wide open and is accessible from the side of the adenosine ribose 5′ diphosphate moiety of NADH. The minor portal is narrower and can be accessible along the long wall of the active-site groove. (b) A close-up view of the active-site groove. NADH and hexanoyl-CoA (HXC) are shown as a ball and stick model. Long slope of the wall of catalytic groove or the minor portal supports hexanoyl-CoA binding. A positively charged cluster near the dimer interface is indicated by the oval shaped region.
Comparison between two ternary complexes put forth four significant differences between these complexes. First, the hexanoyl-CoA in FabG4 adopts an extended conformation, whereas the C16 fattyacyl substrate in InhA has taken a ‘U’-shaped conformation. The ‘U’-shaped conformation is possibly because of the presence of the long C16 fattyacyl chain. Secondly, hexanoyl-CoA accesses the minor portal to reach the active site, unlike the C16 fattyacyl substrate which accesses the major portal. The accessing modes of the two substrates are different probably because the ACP interaction modes with FabI and FabG are different [14, 15]. ACP interacts with FabI via the α-helical subdomain, but ACP interacts with FabG via the α4- and α5-helices of the dimeric interface. Thirdly, the long fattyacyl chain in the C16 substrate is totally isolated from the solvent region and covered by the unusually longer α-subdomain of MtFabI (InhA). In contrast, hexanoyl-CoA bends towards the hydrophobic motifs A306GRT309 of loop I and A124VXA128 of ø6B (Figure 3d). Finally, the loop I in the MtFabI structure, which is mostly populated with hydrophobic residues, bends inwards to interact with the hydrophobic fattyacyl chain, whereas loop I in FabG4 adopts an extended conformation and opens the minor portal to access the CoA-specific substrate. FabG4 and hexanoyl-CoA interaction not only proves that the FabG–substrate interaction is different than that of FabI–substrate interaction, but also reveals detailed interaction between a reductase of the FAS-II pathway and the 4’ phosphopantetheine moiety of either ACP or CoA. The hexanoyl-CoA-bound FabG4 structure was compared with other substrate-bound short-chain dehydrogenase reductase structures. Superimposition of the FabG4 ternary complex on to the mycobacterial 3a, 2β hydroxyl steroid dehydrogenase reductase structures. Superimposition of the FabG4 ternary complex on to the mycobacterial 3a, 2β hydroxyl steroid dehydrogenase ternary complex [38] shows that both the substrates follow the same route to the active site for entry. Other fattyacyl substrate-mimicking complexes such as PEG [poly(ethylene)glycol] are also found near the FabG active site [39], supporting the localization of the CoA substrate.

### Catalytic mechanism inferred from the FabG4 structures

The catalytic mechanism is well studied in LMwFabGs, although the exact reaction mechanism is a matter of debate. Initial velocity, product inhibition and the deuterium kinetic isotope effect have suggested either rapid equilibrium [41] or steady-state [42] random Bi Bi rapid equilibrium kinetic mechanisms. Other reports have shown that an ordered mechanism is followed where NADPH binds first [43, 44]. The hydride-transfer mechanism of FabG4 is not very different from its LMwFabG counterparts because FabG4 possesses the same catalytic residues as LMwFabG. Also their catalytic site architecture is very similar.

The ternary complex of FabG4–NADH–HXC revealed the first structural evidence of the arrangement of the substrates near the FabG4 catalytic site (Figure 7A). The β-carbon of hexanoyl-CoA is sandwiched between the hydride donor C4 carbon of the nicotinamide moiety and the catalytic tyrosine (Figure 7A). Among the four catalytic residues only Tyr416 is situated in close contact with the β-carbon. Catalytic Ser135, however, is involved in hydrogen bonding with thioester carbonyl oxygen to align the substrate for catalysis. On the basis of the structural evidence a possible hydride-transfer mechanism is presented below.

The association of both β-oxoacyl-CoA and NADH with FabG4 starts the sequence of hydride transfer. In NAD the nicotinamide moiety is not planar; therefore, the amine group attached with the C3 atom of the nicotinamide ring can move comparatively freely to interact with the protein. Additionally, the nitrogen atom of the nicotinamide ring participates in a hydrogen-bonding interaction with Tyr360. Tyr360 and Lys368 also help guide the nicotinamide moiety of NADH to orientate in a proper conformation. In this situation the β-oxo carbonyl oxygen transfers the π electron to check whether FabG4 interacts with ACP using an ACP-inhibition study. Because AcpM partly sits on the CoA-binding region it acts as an inhibitor of acetoacetyl-CoA. FabG4 activities were measured in presence of different AcpM concentrations. Inhibition (Figure 6) and dose–response (Supplementary Figure S3 at http://www.biochemj.org/bj/450/bj4500127add.htm) curves clearly shows that AcpM indeed inhibits FabG4 oxoreductase activity where 50% inhibition occurs at a concentration of 31 μM AcpM using 1 mM of acetoacetyl-CoA substrate. Structural superimposition of the N-terminal domain (residues 20–196) on to EcFabG gave an r.m.s.d. value of 1.99 Å over 166 Cα residues. The superimposition also shows that the corresponding arginine residues of EcFabG (Arg179 and Arg182) in FabG4 could be Arg115 and Arg153. These arginine residues, along with Arg154, create a positively charged cluster near the entrance to the catalytic groove (Figure 5). This positively charged cluster might help ACP to dock on FabG4; however, sequence alignment analysis suggested that the constituent arginine residues of the positively charged cluster are not conserved among HMwFabGs. Rather, conserved Arg111 and Lys160 around the same region might undergo a conformational change to facilitate ACP docking with HMwFabGs. Therefore in HMwFabG4 the conserved Arg111 and Lys160 might be responsible for ACP–HMwFabG interaction. The docking results also support the ACP inhibition data. Like the LMwFabG tetramer, surface-charge distributions in two faces of the FabG4 dimer are not symmetrical (Supplementary Figure S4 at http://www.biochemj.org/bj/450/bj4500127add.htm). One face is populated with positively charged clusters harbouring two active sites and the opposite face is populated with mostly negatively charged clusters with islets of positive patches. ACP docks on the active site containing face at the same region where the substrate binds (Figure 6) resulting in ACP inhibition.

### FabG4 can accept both CoA- and ACP-based substrates

For the FAS-II fatty acid synthetic pathway ACP is required to shuttle the elongating fattyacyl chain from one enzyme to another. Nevertheless, there is no specific ACP-recognition motif identified among type-II fatty acid enzymes [15]. ACP–LMwFabG interaction has been studied using NMR spectroscopy. NMR results have shown that in _EcFabG_ Arg179 and Arg182 are two residues important for ACP–LMwFabG interaction [15]. Investigation of the _EcFabG_ structure showed that Arg179 and Arg182 reside on the top of the α4- and α5-helices and can easily be accessed by the ACP. However, ACP cannot access the active site of a particular monomer while sitting on it, although it can provide a substrate to the second monomer and therefore these two monomers must make a dimer along the α4- and α5-helices. Using fluorescence spectroscopy it was shown that in _SrFabG1_, the α4- and α5-helices are involved in dimer formation [39]. Similar results are obtained from the docking studies of ACP with LMwFabG [40]. These observations are further confirmed by our docking results. AcpM docks on the α4- and α5-helices of _MrFabG1_ at the dimeric interface and can provide the elongating fattyacyl substrate to the second monomer (Supplementary Figure S2 at http://www.biochemj.org/bj/450/bj4500127add.htm). On the other hand, the FabG4 dimer mimics a tetrameric assembly of LMwFabG, two of its active sites are accessible from same face. In the FabG4 dimer ACP has to interact with the N-terminal domain of one monomer providing the β-oxoacyl substrate to the C-terminal domain of another monomer. We have attempted to check whether FabG4 interacts with ACP using an ACP-inhibition study. Because AcpM partly sits on the CoA-binding region it acts as an inhibitor of acetoacetyl-CoA. FabG4 activities were measured in presence of different AcpM concentrations. Inhibition (Figure 6) and dose–response (Supplementary Figure S3 at http://www.biochemj.org/bj/450/bj4500127add.htm) curves clearly shows that AcpM indeed inhibits FabG4 oxoreductase activity where 50% inhibition occurs at a concentration of 31 μM AcpM using 1 mM of acetoacetyl-CoA substrate. Structural superimposition of the N-terminal domain (residues 20–196) on to EcFabG gave an r.m.s.d. value of 1.99 Å over 166 Cα residues. The superimposition also shows that the corresponding arginine residues of EcFabG (Arg179 and Arg182) in FabG4 could be Arg115 and Arg153. These arginine residues, along with Arg154, create a positively charged cluster near the entrance to the catalytic groove (Figure 5). This positively charged cluster might help ACP to dock on FabG4; however, sequence alignment analysis suggested that the constituent arginine residues of the positively charged cluster are not conserved among HMwFabGs. Rather, conserved Arg111 and Lys160 around the same region might undergo a conformational change to facilitate ACP docking with HMwFabGs. Therefore in HMwFabG4 the conserved Arg111 and Lys160 might be responsible for ACP–HMwFabG interaction. The docking results also support the ACP inhibition data. Like the LMwFabG tetramer, surface-charge distributions in two faces of the FabG4 dimer are not symmetrical (Supplementary Figure S4 at http://www.biochemj.org/bj/450/bj4500127add.htm). One face is populated with positively charged clusters harbouring two active sites and the opposite face is populated with mostly negatively charged clusters with islets of positive patches. ACP docks on the active site containing face at the same region where the substrate binds (Figure 6) resulting in ACP inhibition.

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Figure 6  AcpM inhibition of FabG4 oxoreductase activity

FabG4 activity is measured at different concentrations of AcpM. The inset shows that AcpM docks at the FabG4 dimeric interface and partly occupies the hexanoyl-CoA (HXA) position. Error bars represent the averages from two independent duplicate experiments.

to the proton attached with Tyr360 hydroxyl oxygen yielding a planar carbocation. The hydride of the C4 nicotinamide ring nearest to the β-carbon is then transferred to this β-carbocation. As a consequence a β-hydroxy fattyacetyl substrate is generated (Figure 7B). This newly implanted chirality of β-carbon then forces the substrate to leave the reaction centre. Tyr360 then enters into proton relay system to replenish its proton deficiency as described earlier [34]. On the other hand the nicotinamide ring adopts a planar conformation. Comparing the FabG4–NADH and FabG4–NAD+–HXC structures we assume that the planar conformation induces a significant conformational change in the pyrophosphate moiety. As a consequence, the interaction between NAD+ and protein switches over from one interacting conformation to another (Figure 7A). The amino acid residues that are primarily involved in the interacting-conformation shift are Arg223 and a part of α′-helix residues (Thr395, Ile393 and Gly391). Arg223 is conserved among FabGs and the G169(F/Y)(I/V/M)XT395 motif is conserved among most NAD(P)H using short-chain dehydrogenase/reductases [33]. The conformational orientation switch results in a reduced NAD+ affinity towards FabG4 and forces NAD+ to leave the reaction site.

FabG4 is a β-oxoacyl reductase

Unlike its LMwFabG counterparts FabG4 utilizes NADH to reduce the β-oxoacyl group to β-hydroxyacyl. FabG4 can also participate in the reverse reaction using NAD+ as a coenzyme. Because β-hydroxyacyl dehydrogenase can also participate in the same reverse reaction the question arises if β-oxoacyl reductase name of FabG4 is a misnomer. We have used acetoacetyl-CoA/NADH and β-hydroxybutyryl-CoA/NAD+ pairs to determine the kinetic parameters for both forward and reverse reaction respectively (Supplementary Figure S4 at http://www.biochemj.org/bj/450/bj4500127add.htm). We found that the catalytic efficiency (kcat/K′) of forward reaction is almost four times than that of the reverse reaction (Table 2). The K′ value, which is indicative of a dissociation constant [45], shows that the value for acetoacetyl-CoA is almost two thirds that of DL-β-hydroxybutyryl-CoA. Therefore the kinetic data is also in agreement with the fact that FabG4 prefers NADH over NAD+. Another important outcome of the kinetic data is the absence of cooperativity in the reverse reaction. This is imperative because the natural substrates are energetically favourable and thus behave in a usual way. Co-operativity among FabG thus implicates that the dehydrogenase reaction does not have the usual characteristics of FabG4. Although substitutive substrates are used to determine the kinetic constants of β-oxoacyl reductases [38], we have not seen any activity with DL-β-hydroxybutyric acid [9]. When using DL-β-hydroxybutyric acid as a substrate, the FabG4 kinetic profiles show only nominal changes in dehydrogenase activity with sufficient increment of substrate concentration. This result implies that the pantetheine moiety of CoA is primarily responsible for efficient catalysis by aligning the substrate near the catalytic site [46]. Comparing the catalytic efficiency of FabG4 with other LMwFabGs indicates that its catalytic efficiency of using CoA substrate is much higher than any other known LMwFabG.

Arg146 and Arg445 of FabG4 are important for intersubunit catalytic domain communication

Oligomerization of FabG contributes to the co-operative behaviour of the enzyme. LMwFabGs display either positive [39,47] or negative [43,48,49] co-operativity depending on their oligomeric state [39]. Fluorescence studies have enabled us to conclude that the positive co-operative behaviour of a LMwFabG stems from the dimeric association along α4- and α5-helices and these helices are responsible for the co-operative nature of FabG [39]. Similarly, in the case of tetrameric LMwFabG, extensive contacts between the catalytic domains of LMwFabG monomers are held responsible for their negative co-operativity [43,49]. Invariably, FabG4 exerts positive co-operativity for both acetoacetyl-CoA and NADH; however, here the two catalytic domains only share C-terminal contacts. Two conserved arginines, Arg146 and Arg445 (Figure 1A), of
Figure 7  Superposition of binary and ternary complexes near the active site and implied catalytic mechanism

(A) The β-carbon of the hexanoyl moiety is sandwiched between the nicotinamide ring and catalytic Tyr360. Only Tyr360 among the catalytic tetrad is in close proximity to the β-carbon. The nicotinamide moiety in NAD⁺ is involved in amine group interaction with Thr395, Gly391, Phe392 and Ile393 of α' subdomain and intramolecule diphosphate. Change in the planarity of the nicotinamide ring is responsible for shift from one interacting conformation to another via altered interaction with α' subdomain residues and NADH diphosphate moiety. (B) The catalytic mechanism inferred from the ternary complex. Tyr360 is responsible for protonation to the β-oxo oxygen resulting in planar β-carbocation. The β-carbocation then accepts the nearest hydrogen from C4 carbon of the nicotinamide ring. Ser347 holds a thioester group to align the substrate.

one monomer point towards the C-terminus of the second monomer and are involved in a hydrogen-bonding network together with conserved structural water (Supplementary Figure S5 at http://www.biochemj.org/bj/450/bj4500127add.htm). To substantiate the role of Arg146 and Arg445 in catalysis and substrate association the R146A, R445A and R146A + R445A mutants were produced using site-directed mutagenesis. The kinetic parameters of recombinant FabG4 and mutants are summarized in Table 2. The $k_{cat}$ value for NADH is reduced indicating multiple conformational states of NADH in one cycle of catalysis [45]. No significant changes in NADH affinity ($K_{NADH}^*$) due to R146A, R445A or double mutant are observed. The catalytic efficiency ($K_{cat}/K_{NADH}^*$) for R146A, R445A and the double mutant is 2.2-, 12- and 26-fold less respectively compared with the wild-type. In case of acetoacetyl-CoA R146A the affinity ($K_{AcAcCoA}^*$) is almost the same as the wild-type. Also, the catalytic efficiency is reduced only 1.2-fold compared with that of the wild-type. The effect of the R146A mutation in acetoacetyl-CoA catalysis is lower compared with NADH catalysis probably because of the huge difference between NADH and acetoacetyl-CoA affinity. However, for R445A or the double mutant both affinity ($K_{AcAcCoA}^*$) and catalytic efficiency ($k_{cat}/K_{AcAcCoA}^*$) of acetoacetyl-CoA are severely compromised. Mutation of Arg445 to an alanine residue perturbs the C-terminal interaction where the conserved C-terminus of HMwFabG is responsible for loop I and II stability. Loop I and II further help align Tyr160 and Lys364 in catalytic orientation [9]. Because there is no direct interaction between NADH and the catalytic loops, the arginine mutations do not affect NADH affinity, but the turnover decreases as the arginine residues are important.
for the correct catalytic orientation of Tyr$^{360}$ and Lys$^{364}$. In contrast, the R146A mutation renders no significant alteration in the $K_{A/C0A}$ value because acetoacetyl-CoA binds comparatively weakly with the protein and Arg$^{366}$ makes weak water-mediated intercatalytic domain interactions. For the reason that NADH binds strongly compared with the acetoacetyl-CoA the decrease in NADH catalysis for the R146A mutation is more prominent than that of acetoacetyl-CoA catalysis. For the R445A mutant, the acetoacetyl-CoA catalysis is severely compromised yielding further reduced activity for the double mutant. Owing to the R445A mutation the rigid interaction of the C-terminus is almost lost and catalytic loops are not stable. As a consequence, loop I which harbours the CoA substrate results in reduced acetoacetyl-CoA affinity.

Even among LMwFabG structures the possible role of C-terminus is predicted [50]. FabG1 sequences among different mycobacterial species are found to possess a specific motif [51]. Initially, the stability of loop I and II is correlated with the presence of a coenzyme [34,51]. Later it was found that the stability of loop II is largely influenced by the particular amino acid located prior to the catalytic serine residue [52].

**Conclusion**

Binary and ternary complexes have revealed the basis of coenzyme and substrate specificity of FabG4, a high molecular weight oxoacyl reductase. The C-terminal residues of FabG4 participate in intercatalytic domain interaction and play a pivotal role in loop I stabilization. Loop I, which is universally present in oxoacyl reductases, is responsible for interaction with phosphopentehine moiety-linked fattyacyl substrates of CoA or ACP. As the substrate–protein interaction in FabG significantly differs from that of FabI interactions, these structural insights can be used to design specific structure-based inhibitors against FabG. Also, the ternary complex sheds light on the catalytic mechanism of FabG. Another important aspect of this work is the identification of FabG4 which utilizes NADH to reduce $\beta$-oxoacyl group to $\beta$-hydroxacyl covalently linked with CoA. Both its ability to use CoA substrate and specificity towards energetically cheaper NADH indicates that FabG4 in *Mycobacterium* may play an important role in substitutive fatty acid biosynthetic pathway during the stressed condition.

**AUTHOR CONTRIBUTION**

Amit Kumar Das initiated the project and designed the work. Debajyoti Dutta performed cloning, purification, crystallization and determination of the structures. Debajyoti Dutta and Sudipta Bhattacharyya did the kinetic experiments. Amlan Roychowthury and Rupam Biswas did the AcpM cloning and AcpM-inhibition assays. Debajyoti Dutta prepared a draft of the paper. Amit Kumar Das analysed the data and wrote the final paper.

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Crystal structure of hexanoyl-CoA bound to β-ketoacyl reductase FabG4 of Mycobacterium tuberculosis

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Cloning, overexpression and purification of AcpM of M. tuberculosis

*M. tuberculosis* acpM was amplified by PCR using *M. tuberculosis* H37Rv genomic DNA as the template with the primer pair 5'-GATGGATCCGTGCTGACGTCAAGGAAG-3' (BamHI site underlined) and 5'-CTTAAGCTTTCACCTGGACTGCCATCAAGC-3' (HindIII site underlined). The amplified product was ligated downstream of a His6-encoding sequence into the pQE30 overexpression vector (Qiagen), which had been previously treated with the BamHI and HindIII restriction enzymes. The construct was used to transform chemically competent *E. coli* M15 (pREP4) cells and subsequently selected on ampicillin (100 μg/ml) and kanamycin (25 μg/ml) plates. The positive clones were verified by DNA sequencing.

The recombinant strain was grown at 37°C in a rotary shaker (200 rev./min) in 2 litres of LB broth with antibiotics. The expression of acpM was induced by the addition of 100 μM IPTG (isopropyl β-D-thiogalactopyranoside) until *D*600 reached 0.6 and further incubated for 4 h. Cells were harvested at 8000 rev./min for 10 min. Harvested cells were resuspended with resuspension buffer [10 mM Tris/HCl (pH 7.9), 300 mM NaCl and 10 mM imidazole] supplemented with protease inhibitor cocktail (0.1 mM each leupeptin, pepstatin and aprotinin and 0.2 mM PMSF). *E. coli* cells were lysed using ultrasonication and centrifuged at 22 800 g for 40 min and the supernatant was collected and loaded on to a Ni-Sepharose high performance affinity matrix (GE Healthcare Biosciences) preequilibrated with resuspension buffer. After loading the supernatant, the column was extensively washed with resuspension buffer. Buffer A [10 mM Tris/HCl (pH 7.9), 300 mM NaCl and 30 mM imidazole] was subsequently passed through the column to remove any non-specifically bound contaminant. The protein was finally eluted with elution buffer [10 mM Tris/HCl (pH 7.9), 300 mM NaCl and 100 mM imidazole]. The eluted protein was subjected to buffer exchange in a vivaspin 5 kDa MWCO (molecular mass cut-off; GE Healthcare Biosciences) filter by buffer B [30 mM Tris/HCl and 50 mM NaCl (pH 7.9)]. It was then loaded into a Q Sepharose (Pharmacia Biotech) anion-exchange column preequilibrated with buffer B. After washing with buffer B the protein was eluted using a linear gradient of buffer C [30 mM Tris/HCl and 1 M NaCl (pH 7.9)]. Gel-filtration chromatography was done using superdex 75 prep-grade matrix in a 16/70 column (GE Healthcare Biosciences) on an AKTAprime plus system (GE Healthcare Biosciences) with buffer D [10 mM Tris/HCl and 50 mM NaCl (pH 7.9)]. Each fraction of 2 ml was collected at a flow rate of 1 ml/min. The fractions containing the desired protein were pooled together and kept for further use. Protein purity was checked using SDS/PAGE (12% gel).

Figure S1 Superimposition of the NADP-bound structures

Superimposition of the NADP-bound structures of EcFabG (blue), MtFabG1 (magenta), BnFabG (lime green), SaFabG1 (yellow) on to MtFabG4 (red) showing the structural difference around 2′ adenosine ribose phosphate. Asp244 of the β2B–α3B loop is primarily important for NADH preference. Orientation of Arg223 on the β1B–α1B loop is responsible for different conformations of pyrophosphate moiety of NAD(P).
Figure S2  Computational docking of major AcpM on MtFabG1 and FabG4

(a) AcpM sits on the C-terminal top of the $\alpha_4$- and $\alpha_5$-helices of the MtFabG1 tetramer to access the active site of the second monomer. (b) AcpM docks the hexanoyl-CoA (HXC)-binding site where it can interact with the N-terminal domain of one monomer and can extend the elongating $\beta$-oxoacyl fattyacyl substrate to the active site (C-terminal domain) of the second monomer.

Figure S3  Dose–response curve of AcpM inhibition of FabG4 activity

Error bars represent data range from two independent duplicate experiments.
The distribution of surface charge is different for FabG4 (upper panels), but symmetric for LMwFabG (lower panels).
Figure S5  Kinetic characterization of FabG4 and its R146A, R445A mutants

(a) NADH is varied keeping acetoacetyl-CoA in saturating condition. The wild-type enzyme (■), R146A mutant (▼), R445A mutant (▲) and double mutant (●) are shown and fitted to the Hill equation. (b) Acetoacetyl-CoA is varied keeping NADH in saturating condition. The wild-type enzyme (■), R146A mutant (▼), R445A mutant (▲) and double mutant (●) are shown and fitted to the Hill equation. (c) and (d) represents the velocity curves of the reverse reactions. Error bars represent data range from two independent duplicate experiments.
Structure–function relationship of HMwFabG

**Figure S6  The C-terminal hydrogen bonding network**

Two different monomers are indicated by two different colours. Arg\textsuperscript{146} and Arg\textsuperscript{445} of one monomer hold the C-terminus of Ala\textsuperscript{454} of another monomer. The interaction is assisted by some conserved water network. Arg\textsuperscript{445} of one monomer makes direct hydrogen bond with Ala\textsuperscript{454} of second monomer, whereas Arg\textsuperscript{146} makes a water-mediated interaction.

**Table S1  Primers used to amplify the MtFabG4 mutants**

Restriction sites are underlined.

<table>
<thead>
<tr>
<th>Open reading frame</th>
<th>Primer designation</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>FP0242C (SacI)</td>
<td>5'-CGAGCTCATGG6GTCCAGGATCGTTTTGGCCAG-3'</td>
</tr>
<tr>
<td></td>
<td>N_242FP (BamHI)</td>
<td>5'-CGGGATCCGGTCCAGGATCGTTTTGGCCAG-3'</td>
</tr>
<tr>
<td></td>
<td>C_242RP (HindIII)</td>
<td>5'-CCCAAGCTTTCACGCGCCGATCATGGCC-3'</td>
</tr>
<tr>
<td>R146A mutation</td>
<td>R146AMRP</td>
<td>5'-CGGAACCGCGGAAGCTCAGGGTACGTTTTGGCCAG-3'</td>
</tr>
<tr>
<td></td>
<td>R146AMFP</td>
<td>5'-CGGAACCGCGGAAGCTCAGGGTACGTTTTGGCCAG-3'</td>
</tr>
<tr>
<td>R445A mutation</td>
<td>R445AmRP</td>
<td>5'-CAGACAGCAATGACGTTGCCGGTCACCG-3'</td>
</tr>
<tr>
<td></td>
<td>R445AmFP</td>
<td>5'-CGGTGACCGGCAACGTCATTGCTGTCTG-3'</td>
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**Table S2  NADH and FabG4 interaction distances and water molecules**

Protein residues are in chain A.

<table>
<thead>
<tr>
<th>Protein residue</th>
<th>NADH</th>
<th>Water residue</th>
<th>Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu266/O</td>
<td>NA</td>
<td>adenosine part/N1A</td>
<td>3.4</td>
</tr>
<tr>
<td>Val268/N</td>
<td>NA</td>
<td>adenosine part/N1A</td>
<td>3.1</td>
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<tr>
<td>Asp244/OD2</td>
<td>NA</td>
<td>ribose/OH1</td>
<td>3.4</td>
</tr>
<tr>
<td>Asp244/OD2</td>
<td>NA</td>
<td>ribose/OH1</td>
<td>3.0</td>
</tr>
<tr>
<td>Arg223/NH1</td>
<td>NA</td>
<td>ribose/OH1</td>
<td>2.7</td>
</tr>
<tr>
<td>Gly227/O</td>
<td>NA</td>
<td>ribose/OH1</td>
<td>2.8</td>
</tr>
<tr>
<td>Ile225/N</td>
<td>NA</td>
<td>ribose/OH1</td>
<td>3.5</td>
</tr>
<tr>
<td>Asn295/O</td>
<td>NA</td>
<td>nicotinamide ring/N1</td>
<td>2.8</td>
</tr>
<tr>
<td>Lys296/NZ</td>
<td>NA</td>
<td>nicotinamide ring/N1</td>
<td>3.0</td>
</tr>
<tr>
<td>Lys296/NZ</td>
<td>NA</td>
<td>nicotinamide ring/N1</td>
<td>3.0</td>
</tr>
<tr>
<td>Thr398/OG1</td>
<td>NA</td>
<td>nicotinamide amide/NH2</td>
<td>3.0</td>
</tr>
</tbody>
</table>

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Table S3  Hexanoyl-CoA (HXC), FabG4 and NAD interaction distances and and water molecules

<table>
<thead>
<tr>
<th>Protein residue</th>
<th>HXC</th>
<th>NAD †</th>
<th>Water</th>
<th>Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu^{151}/O*</td>
<td>HXC/adenosine amide/N6</td>
<td>–</td>
<td>–</td>
<td>3.6</td>
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<tr>
<td>Arg^{153}/NH1*</td>
<td>HXC/adenosine ring/NH1</td>
<td>–</td>
<td>–</td>
<td>2.9</td>
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<tr>
<td>Asn^{366}/ND2</td>
<td>HXC/5′ diphosphate/O6</td>
<td>–</td>
<td>W^{668}/O</td>
<td>2.7</td>
</tr>
<tr>
<td>Asn^{366}/ND2</td>
<td>HXC/5′ diphosphate/O21</td>
<td>–</td>
<td>–</td>
<td>3.1</td>
</tr>
<tr>
<td>Leu^{393}/N</td>
<td>HXC/5′ diphosphate/O21</td>
<td>–</td>
<td>–</td>
<td>2.6</td>
</tr>
<tr>
<td>Lys^{392}/NZ</td>
<td>HXC/5′ diphosphate/O22</td>
<td>–</td>
<td>–</td>
<td>2.7</td>
</tr>
<tr>
<td>Leu^{393}/CZ†</td>
<td>HXC/pantethine/CPA</td>
<td>–</td>
<td>–</td>
<td>3.0</td>
</tr>
<tr>
<td>Asp^{394}/OD2</td>
<td>HXC/pantethine/NP1</td>
<td>–</td>
<td>–</td>
<td>3.4</td>
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<tr>
<td>Asn^{394}/ND2</td>
<td>HXC/pantethine/OP1</td>
<td>–</td>
<td>–</td>
<td>3.2</td>
</tr>
<tr>
<td>Ser^{347}/OG</td>
<td>HXC/thioester/OM2</td>
<td>–</td>
<td>W^{632}/O</td>
<td>3.2</td>
</tr>
<tr>
<td>Thr^{299}/CG2†</td>
<td>HXC/fattyacyl chain/CM4</td>
<td>–</td>
<td>W^{652}/O</td>
<td>2.7</td>
</tr>
<tr>
<td>Thr^{299}/CG2†</td>
<td>HXC/fattyacyl chain/CM5</td>
<td>–</td>
<td>W^{652}/O–W^{652}/O</td>
<td>2.6</td>
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<tr>
<td>Gly^{301}/CA†</td>
<td>HXC/fattyacyl chain/CM6</td>
<td>–</td>
<td>–</td>
<td>3.6</td>
</tr>
<tr>
<td>Gly^{301}/CA‡</td>
<td>HXC/fattyacyl chain/CM3</td>
<td>NAD+ /nicotinamide moiety C4/C4N</td>
<td>–</td>
<td>4.5</td>
</tr>
<tr>
<td>Tyr^{361}/OH‡</td>
<td>HXC/fattyacyl chain/CM3</td>
<td>–</td>
<td>–</td>
<td>4.0</td>
</tr>
</tbody>
</table>

*Residues are from symmetry mate (the second monomer of FabG4 dimer).
†Hydrophobic interaction.
‡Active site of hexanoyl-CoA–NAD.