Mycolic acid biosynthesis and enzymic characterization of the β -ketoacyl-ACP synthase A-condensing enzyme from *Mycobacterium tuberculosis*

Laurent KREMER^{*1,2}, Lynn G. DOVER^{*1}, Séverine CARRÈRE[†], K. Madhavan NAMPOOTHIRI^{*}, Sarah LESJEAN[‡], Alistair K. BROWN^{*}, Patrick J. BRENNAN[§], David E. MINNIKIN^{||}, Camille LOCHT[‡] and Gurdial S. BESRA^{*3}

^{*}Department of Microbiology and Immunology, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, U.K., [†]CNRS FRE2369, Institut Pasteur de Lille/IBL, Lille, France, [‡]INSERM U447, Institut Pasteur de Lille/IBL, Lille, France, [§]Department of Microbiology, Colorado State University, Fort Collins, CO 80523-1677, U.S.A., and ^{||}Department of Chemistry, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, U.K.

Mycolic acids consist of long-chain α -alkyl- β -hydroxy fatty acids that are produced by successive rounds of elongation catalysed by a type II fatty acid synthase (FAS-II). A key feature in the elongation process is the condensation of a two-carbon unit from malonyl-acyl-carrier protein (ACP) to a growing acyl-ACP chain catalysed by a β -ketoacyl-ACP synthase (Kas). In the present study, we provide evidence that *kasA* from *Mycobacterium tuberculosis* encodes an enzyme that elongates *in vivo* the meromycolate chain, in both *Mycobacterium smegmatis* and *Mycobacterium chelonae*. We demonstrate that KasA belongs to the FAS-II system, which utilizes primarily palmitoyl-ACP rather than short-chain acyl-ACP primers. Furthermore, in an *in vitro*

condensing assay using purified recombinant KasA, palmitoyl-AcpM and malonyl-AcpM, KasA was found to express Kas activity. Also, mutated KasA proteins, with mutation of Cys¹⁷¹, His³¹¹, Lys³⁴⁰ and His³⁴⁵ to Ala abrogated the condensation activity of KasA *in vitro* completely. Finally, purified KasA was highly sensitive to cerulenin, a well-known inhibitor of Kas, which may lead to the development of novel anti-mycobacterial drugs targeting KasA.

Key words: acyl-carrier protein, cerulenin, fatty acid, type II fatty acid synthase.

INTRODUCTION

Tuberculosis is regarded as one of the world's most deadly infectious diseases [1]. The recent emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* poses a serious threat to tuberculosis control programmes worldwide. Understanding the mechanisms of resistance to the anti-mycobacterial drugs currently available [2] and the discovery of new drug targets are necessary for the treatment of tuberculosis.

Mycolic acids are long-chain α -alkyl- β -hydroxy fatty acids (R-CH[OH]-CH[R']-COOH) unique to mycobacteria and related taxa and are believed to play a crucial role in the architecture of the mycobacterial cell envelope [3]. R represents the meromycolate chain consisting of 50–56 carbon atoms and R' is a shorter aliphatic branch possessing 22–26 carbon atoms [3]. Fatty acid biosynthesis in mycobacteria involves two distinct synthases, namely, type I fatty acid synthase (FAS-I) and type II fatty acid synthase (FAS-II). The former is a single polypeptide with multiple catalytic activities generating short-chain acyl-CoA esters. Mycobacterial FAS-II is analogous to other bacterial FAS-II elongation systems and consists of dissociable enzyme components [4,5], which act upon a substrate bound to an acyl carrier protein (ACP), designated as AcpM in *M. tuberculosis* [6]. Recently, it has been shown that mtFabH [a distinct β -ketoacyl-ACP synthase (Kas)] forms a pivotal link between the type I and type II FAS elongation systems in *M. tuberculosis* [7]. Indeed, the preferred substrates for mtFabH consist of lauroyl (C₁₂)-CoA and myristoyl (C₁₄)-CoA, which are produced by FAS-I, and following condensation and a complete cycle of fatty acid

biosynthesis, generate C₁₄-AcpM and palmitoyl (C₁₆)-AcpM respectively. Mycobacterial FAS-II, unlike other bacterial type II FASs, is incapable of *de novo* fatty acid biosynthesis [5,8] but appears to elongate C₁₄-AcpM and C₁₆-AcpM preferentially to long-chain fatty acids ranging from 24 to 56 carbon atoms in length [9].

In this report, we have extended our previous studies on mycolic acid biosynthesis by providing further *in vivo* evidence that KasA is an integral part of the mycobacterial type II FAS elongation system. We also demonstrate that *in vitro*, KasA catalyses the key elongation–condensation steps in mycolic acid biosynthesis, condensing malonyl-AcpM with C₁₆-AcpM. In addition, cerulenin, which possesses anti-mycobacterial activity [10] and is a well-known Kas inhibitor in other bacteria [4], possesses potent inhibitory properties when compared with *in vitro* KasA condensation activity. Thus KasA represents a prospective drug target yet to be examined in drug discovery programmes with the possibility of leading to novel chemotherapeutic agents targeting *M. tuberculosis*.

MATERIALS AND METHODS

Strains, plasmids and media

Strains and plasmids used in the present study are shown in Table 1. Luria broth (Difco) and Luria agar containing 25 μ g/ml kanamycin (Sigma) were used for the growth of *Escherichia coli* [11]. *Mycobacterium smegmatis* (MSG) strains carrying pMV261 or its derivatives were grown in liquid culture using Sauton medium supplemented with 25 μ g/ml kanamycin at

Abbreviations used: ACP, acyl-carrier protein; AcpM, ACP mycobacteria; C₁₂, lauroyl; C₁₄, myristoyl; C₁₆, palmitoyl; DIG, digoxigenin; mtFabD, malonyl-CoA:ACP transacylase; FabH, mtFabH, β -ketoacyl-ACP synthase III; FAME, fatty acid methyl ester; FAS, fatty acid synthase; INH, isoniazid; Kas, β -ketoacyl-ACP synthase; MAME, mycolic acid methyl ester; MSG, *Mycobacterium smegmatis*; MTB, *Mycobacterium tuberculosis*.

¹ The two authors contributed equally to this work.

² Present address: INSERM U447, Institut Pasteur de Lille, 1 rue du Pr. Calmette, 59019 Lille, France.

³ To whom correspondence should be addressed, at the present address: School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K. (g.besra@bham.ac.uk).

Table 1 Strains and plasmids used in this study

Strain/plasmid	Genotype/phenotype/description	Reference/source
Strains		
<i>E. coli</i> XL1-Blue	F ['] ::Tn10 <i>proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^q Δ (<i>lacZ</i>) <i>M15/recA1</i> <i>endA1</i> <i>Gyr96</i> (Nal ^r) <i>thi</i> <i>hsdR17</i> (<i>r</i> _k ⁺) <i>supE44</i> <i>relA1</i> <i>lac</i>	Biolabs
<i>E. coli</i> C41(DE3)	BL21(DE3) mutant strain	[14]
<i>M. smegmatis</i> mc ² 155	Electroporation-efficient mutant of mc ⁶	[30]
<i>M. chelonae</i>	Wild-type	ATCC 19536
<i>M. fortuitum</i>	Wild-type	PHLS, U.K.
Plasmids		
pMV261	<i>E. coli</i> –mycobacteria shuttle vector	[31]
pMV261:: <i>kasA</i> _MTB	pMV261 containing <i>M. tuberculosis</i> H37Rv <i>kasA</i>	[10]
pMV261:: <i>kasA</i> _MSG	pMV261 containing <i>M. smegmatis kasA</i>	This work
ET28a	<i>E. coli</i> expression vector	Novagen
pET28a:: <i>kasA</i>	pET28a containing <i>M. tuberculosis</i> H37Rv <i>kasA</i>	This work
pET28a:: <i>acpM</i>	pET28a containing <i>M. tuberculosis</i> H37Rv <i>acpM</i>	[15]
pET28a:: <i>mtfabD</i>	pET28a containing <i>M. tuberculosis</i> H37Rv <i>mtfabD</i>	[15]

37 °C. Recombinant *Mycobacterium chelonae* strains were grown under gentle shaking in a Sauton medium containing 120 µg/ml kanamycin at 30 °C. Middlebrook 7H10 agar supplemented with 10% (v/v) oleic acid/albumin/dextrose/catalase enrichment (Difco) was used for growth on solid media and plating transformations of mycobacteria. Selections for mycobacteria were performed using a solid medium containing kanamycin [25 µg/ml for *M. smegmatis* (3–4 days at 37 °C) or 120 µg/ml for *M. chelonae* (5–7 days at 30 °C)].

DNA manipulation, cloning and sequencing

Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim and Vent-DNA polymerase was purchased from New England Biolabs. All DNA manipulations were performed using standard protocols, as described by Sambrook et al. [11]. Analysis of plasmids from mycobacteria was achieved by electrodution in *E. coli* as described previously [12]. The original *M. tuberculosis kasA* and *kasB* were isolated from *M. tuberculosis* H37Rv genomic DNA [10]. Analogous *M. smegmatis* genes were obtained by screening a genomic library of this organism consisting of a partial *Sau3A* library in pSK (a gift from K. Duncan, GlaxoSmithKline). The library was probed independently with *M. tuberculosis kasA* and *kasB*, labelled with digoxigenin (DIG)–dUTP. The PCR DIG Probe Synthesis Kit (Boehringer Mannheim) was used to label probes with the following specific primers. PCR amplification of *M. tuberculosis kasA* was performed using *M. tuberculosis* genomic DNA in the presence of the upstream primer 5'-GTC AGC CTT CCA CCG CTA ATG-3' and the downstream primer 5'-GCG AAT TCG TGC TTC AGT AAC G-3', whereas amplification of *kasB* used the upstream primer 5'-GGG TAC CAC CAC TTG CGG GGG CGA GT-3' and the downstream primer 5'-GGG GGC CAA GCT TGT CAT CGC AGG TCT-3'. Prehybridization and hybridization were performed at 42 and 50 °C respectively using the DIG Easy Hyb buffer and detected using a DIG luminescence detection kit (Boehringer Mannheim) according to the manufacturer's recommendations. DNA sequencing was performed using the T7 and T3 primers and overlapping clones chosen to reconstitute the entire *M. smegmatis kasA-kasB* sequence.

Cloning of *M. smegmatis kasA* and expression in *M. smegmatis*

PCR amplification of *kasA* was performed using *M. smegmatis* genomic DNA in the presence of the upstream primer P1 5'-TGA CCA GGC CTT CCA CTG CCA ACG GCG GTT AC-3' and the downstream primer P2 5'-GGA ATT CCG CTC TGG CCG TCG AGC AGC TTC TTC-3'. P2 contains an *EcoRI* restriction site (underlined). The 1407 bp fragment corresponding to the *kasA* coding sequence was then digested by *EcoRI* and ligated into the previously *MluNI/EcoRI*-restricted pMV261. The resulting plasmid, designated pMV261::*kasA*_MSG, was used to transform *M. smegmatis*.

Effect of KasA overexpression in relation to mycolic acid and fatty acid profiles in mycobacteria

[1,2-¹⁴C]Acetate (1 µCi/ml, 50–62 mCi/mmol; Amersham, U.K.) was added to mid-log phase mycobacterial cultures, followed by a further 4–6 h incubation at 37 °C for *M. smegmatis*, or an additional 8 h incubation at 30 °C for *M. chelonae*. The ¹⁴C-labelled cells were harvested by centrifugation at 2000 g and washed two times with PBS. The washed cells were then subjected to alkaline hydrolysis and methylation and the resulting mixture of fatty acid methyl esters (FAMES) and mycolic acid methyl esters (MAMES) was further subjected to TLC/autoradiography as described previously [10].

Preparation of cytosolic enzyme fraction: FAS-I and FAS-II assays

The 40–80%-saturated ammonium sulphate precipitate, containing FAS-I and FAS-II activities, was collected after centrifugation and dissolved in 3 ml of buffer (100 mM potassium phosphate, pH 7.0/1 mM EDTA/1 mM dithiothreitol/5 mM MgCl₂) and dialysed overnight as described previously [13]. FAS-I and FAS-II assays were performed as described previously [13].

Expression and purification of *M. tuberculosis* His-tagged KasA

KasA was amplified from genomic DNA isolated from *M. tuberculosis* H37Rv as described previously [10]. The 1260 bp PCR product was ligated into pET28a (Novagen) restricted with *NdeI* and filled-in with the Klenow enzyme previously to create blunt ends. The DNA insert was sequenced to verify the absence of PCR artifacts. The resulting plasmid, designated as pET28a::*kasA*, was used to transform *E. coli* C41(DE3) [14]. An overnight pre-culture of C41(DE3) pET28a::*kasA* was used to inoculate 1 litre of Terrific Broth, which was incubated at 37 °C to an *A*₆₀₀ = 1.2 and then induced with 1 mM isopropyl-β-D-thiogalactoside. Growth was continued for 12–16 h at 16 °C and the cells were harvested by centrifugation. The cell pellet was resuspended in a buffer (20 mM phosphate buffer, pH 7.4/0.5 M NaCl/100 mM imidazole) containing DNase, RNase, complete protease inhibitor-cocktail tablets (Roche) and 0.1 mg/ml of lysozyme (Sigma). Bacteria were disrupted and the resulting extract centrifuged at 27000 g for 60 min at 4 °C. The supernatant was collected and applied on to a Ni²⁺-charged His-Trap column (1 ml; Pharmacia). The column was washed extensively with 20 mM phosphate buffer, pH 7.4/0.5 M NaCl/100 mM imidazole and eluted with a stepwise gradient of imidazole (150, 200, 300 and 500 mM). Fractions (1 ml) were collected and the presence of KasA detected by SDS/PAGE [10% (w/v) gel]. Fractions containing pure KasA were pooled, dialysed against

50 mM Tris/HCl, pH 7.5, containing 10% (v/v) glycerol and stored at -20°C .

Expression and purification of *M. tuberculosis* AcpM and mtFabD (malonyl-CoA:ACP transacylase) in *E. coli*

Both *acpM* and *mtFabD* were cloned into pET28a, generating pET28a::*acpM* and pET28a::*mtfabD* respectively [15]. For overproduction of the recombinant proteins, vectors were then transformed into *E. coli* C41(DE3) and purification of recombinant holo-AcpM, C₁₆-AcpM and mtFabD was achieved using conditions described previously [15].

Site-directed mutagenesis of KasA

A series of site-directed mutants were obtained using pET28a::*kasA* as template for the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Cambridge, U.K.) with the following primers: Cys¹⁷¹ mutated to Ala, 5'-CCC CGG TGT CGG CCG CTT CGT CGG GCT CGG-3' and 5'-CCG AGC CCG ACG AAG CGG CCG ACA CCG GGG-3'; His³¹¹ mutated to Ala, 5'-GAC CAC GTC AAC GCG GCC GGC ACG GCG ACG-3' and 5'-CGT CGC CGT GCC GGC CGC GTT GAC GTG GTC-3'; Lys³⁴⁰ mutated to Ala, 5'-GCG GTG TAC GCG CCG GCG TCT GCG CTG GGC CAC-3' and 5'-GTG GCC CAG CGC AGA CGC CGG CGC GTA CAC CGC-3'; His³⁴⁵ mutated to Ala, 5'-GAA GTC TGC GCT GGG CGC CTC GAT CGG CGC G-3' and 5'-CGC GCC GAT CGA GGC GCC CAG CGC AGA CTT C-3'. All mutant clones used for enzyme purification were verified by automated DNA sequencing.

Kas assay

Mycobacterial KasA assay was performed according to the condensation assays developed for *E. coli* Kas [16,17]. Initially, assay components were mixed together in a batch fashion. The amounts stated correspond to those of a single reaction, which was scaled up proportionately to the number of assays performed. Holo-AcpM (40 μg) was incubated on ice for 30 min with β -mercaptoethanol (0.5 μmol) in a total volume of 40 μl . [2-¹⁴C]Malonyl-CoA (6.78 nmol, 1.66 kBq; Amersham), mtFabD (40 ng) and 25 μl of 1 M potassium phosphate buffer, pH 7.0, were added. The reaction mixture was allowed to stand at 37°C for 20 min to allow the mtFabD-catalysed transacylation of holo-AcpM with malonyl-CoA to reach equilibrium. C₁₆-AcpM (22.5 μg) was added to obtain a final volume of 89 μl . The reaction mixture was then dispensed into 1.5 ml microcentrifuge tubes, according to single assay mixtures. In some instances, cerulenin (up to 10 μg) dissolved in 1 μl of ethanol, or ethanol alone, was added. A 10 μl aliquot of KasA (0.25 μg) was then added and the mixture kept at 37°C for 1 h. The reaction mixture was quenched by the addition of 2 ml NaBH₄-reducing solution converting the β -keto-C₁₈-AcpM product into a C₁₈-1,3-diol. The reducing solution, which was prepared afresh, comprised 5 mg/ml NaBH₄ in 0.1 M K₂HPO₄, 0.4 M KCl and 30% (v/v) tetrahydrofuran [16,17]. The reaction was left at 37°C for at least 1 h. The completely reduced β -ketoacyl product was extracted two times with 2 ml of water-saturated toluene. The combined organic phases from both extractions were pooled and washed with 2 ml of toluene-saturated water. The organic layer was removed and dried under a stream of nitrogen in a scintillation vial. The reduced product was then quantified by liquid scintillation counting using 5 ml of EcoScintA (National Diagnostics, Hull, U.K.).

RESULTS

Overexpression of KasA from *M. tuberculosis* is associated with a decrease in α' -mycolate production

To address the question whether KasA participates in meromycolate elongation, *M. smegmatis* was transformed with *kasA* from *M. tuberculosis* H37Rv. The effect of KasA overproduction on mycolic acid composition was then analysed by TLC after *in vivo* labelling of mycobacterial FAMES and MAMES with [1,2-¹⁴C]acetate. TLC analysis revealed a significant reduction of the α' -subclass in *M. smegmatis* overexpressing KasA (pMV261::*kasA*_MTB; MTB, *M. tuberculosis*) when compared with the control strain carrying the empty pMV261 (Figure 1A). In a compensatory fashion, overexpression of KasA led to an increase of α - and epoxymycolates (Figure 1A). To determine whether this effect was restricted only to *M. smegmatis* as a host or extended to other mycobacterial species, *M. chelonae* was transformed either with the control plasmid pMV261 or with pMV261::*kasA*_MTB.

As shown in Figure 1(B), overexpression of KasA in *M. chelonae* resulted in an increase of the α/α' ratio. Similar results were also obtained when pMV261::*kasA*_MTB was used to transform *M. fortuitum* (results not shown), another fast-growing pathogenic mycobacterial species. These results suggest that overexpression of the *M. tuberculosis* KasA protein consistently increases the α/α' ratio in different mycobacterial species, suggesting that KasA participates in the *in vivo* elongation process of the meromycolic chain. Although, α' -mycolic acids can be considered as precursors of the α -mycolic acids, it is not known why these precursors accumulate in some mycobacterial species, such as *M. smegmatis*. Possible explanations could be that KasA activity may be deficient in these species due to the absence of *kasA*, which seems unlikely, or possibly due to a defect in KasA expression or regulation. Therefore we cloned and sequenced the genetic locus surrounding the *kasA* gene from *M. smegmatis*.

Cloning of *kasA* and *kasB* from *M. smegmatis*

Recently, it has been demonstrated that two other genes, *mabA* (β -ketoacyl-ACP reductase) and *inhA* (enoyl-ACP reductase) are involved in mycolic acid biosynthesis and constitute a single operon in *M. tuberculosis* and *M. bovis* BCG [18]. In contrast, in *M. smegmatis*, *mabA* and *inhA* are transcribed independently [18]. In *M. tuberculosis*, *kasA* and *kasB* belong to the same operon, as depicted in Figure 2(A). However, whether *kasA* and *kasB* belong to the same operon or to separate transcription units in *M. smegmatis* is not known. This was investigated by probing a *M. smegmatis* genomic library with *M. tuberculosis* *kasA* and *kasB*. Overlapping plasmids were analysed by restriction analysis and DNA sequencing, allowing us to determine the sequences of *kasA* and *kasB* as well as partial DNA regions of the adjacent genes. Interestingly, the *kasA* open-reading frame from *M. tuberculosis* and *M. smegmatis* display exactly the same length (1248 bp, 416 amino acids), whereas the *kasB* open-reading frame from *M. smegmatis* is shorter (1251 bp, 417 amino acids) than its *M. tuberculosis* counterpart (1314 bp, 438 amino acids). The DNA region between the end of *kasA* and the beginning of *kasB* from *M. smegmatis* (29 bp) was similar to that of *M. tuberculosis* (30 bp). This indicates that, in contrast with *mabA* and *inhA*, *kasA* and *kasB* are adjacent genes that belong to the same transcriptional unit in both *M. tuberculosis* and *M. smegmatis*. Moreover, sequence analysis revealed that *kasA* was located directly downstream of the ACP gene (*acpM*), whereas *kasB* was found directly upstream of *accD6* encoding an

Figure 1 Normal-phase TLC of FAMES and MAMES in mycobacteria overproducing KasA from *M. tuberculosis*

Mycobacteria were grown in liquid medium and labelled with 1 μ Ci/ml [1,2- 14 C]acetate. Labelled lipids were extracted and resolved by TLC. Autoradiograms were obtained after exposure to Kodak X-Omat AR films. PhosphorImager was used to quantify the proportion of the different mycolic acid subclasses. Equal counts (200 000 c.p.m.) were loaded in each lane. (A) *M. smegmatis* harbouring either pMV261 (empty plasmid) or pMV261::kasA_MTB. The proportion of α' and α +epoxy mycolates was 24 and 76% in the control strain, and 10 and 90% in the strain overproducing KasA. (B) *M. chelonae* carrying either pMV261 or pMV261::kasA_MTB. The proportion of α' and α mycolates was 37 and 63% in the control strain, and 22 and 78% in the strain overproducing KasA. (C) *M. smegmatis* carrying either pMV261 or pMV261::kasA_MSG.

acetyl-/propionyl-CoA carboxylase. Except for *mtfabD* (the first gene of the operon in *M. tuberculosis*, encoding a malonyl-CoA-AcpM transacylase [15]), which has not been sequenced in the present study, our results (not shown) suggest a full conservation of the operon organization in *M. smegmatis*. Further, we investigated whether the *M. smegmatis* KasA protein was active *in vivo* in elongating α' -mycolic acids to α -mycolic acids.

Alteration of the mycolic acid profile in mycobacteria overexpressing KasA from *M. smegmatis*

Given the well-conserved genomic organization of the operon in *M. tuberculosis* and *M. smegmatis* and the high homology of KasA in both species, overexpression of KasA from *M. smegmatis* would probably have the same effect as overexpression of the *M. tuberculosis* protein. Indeed, as shown by TLC analysis in Figure 1(C), *M. smegmatis* overexpressing its homologous gene (pMV261::kasA_MSG) presents a significant reduction of the α' -subclass when compared with the control strain (pMV261). This suggests that the gene product from *M. smegmatis* exhibits the same activity as its *M. tuberculosis* counterpart. Thus KasA participates in *in vivo* meromycolate chain extension.

FAS-II activity in a KasA-enriched cytosolic fraction

We have shown previously that KasA was present in the 40–80 % saturated ammonium sulphate fraction isolated from *M. bovis* BCG overexpressing KasA [10]. When the enriched fraction derived from *M. smegmatis* pMV261 was assayed for fatty acid elongation under conditions that were physiologically relevant, the optimum reaction conditions were obtained in the presence of C_{14} -CoA and C_{16} -CoA, presumably following transacylation to AcpM. This is consistent with the fact that KasIII (mtFabH), which is the pivotal link between the FAS-I and FAS-II in

mycobacteria [7], possesses a clear preference for C_{12} -CoA and C_{14} -CoA. The former substrates are converted by mtFabH followed by a complete cycle of FAS-II into C_{14} -ACP and C_{16} -ACP respectively, the preferred substrates for further elongation via KasA and FAS-II. It is clear that FAS-II has a diminished preference for long-chain acyl-ACP substrates (C_{18} or C_{20}), and almost no specificity for short-chain acyl-ACPs (Figure 3) following *in vitro* transacylation to AcpM. The FAS-II elongation activity was also assessed with the enriched cytosolic fraction isolated from *M. smegmatis* (pMV261::kasA_MTB) under conditions that were physiologically relevant. As shown in Figure 3, although the substrate specificity was conserved, the specific activity was enhanced significantly regardless of the primer used. This indicates that KasA produced in *M. smegmatis* is active enzymically and represents a key enzyme within FAS-II. To characterize the compounds synthesized by FAS-II, FAMES were analysed by C_{18} reversed-phase TLC. The inset (Figure 3) illustrates the chain-length distribution of the FAMES produced for primers C_{12} -CoA/ACP and C_{14} -CoA/ACP and shows that the yield decreases sharply with increasing chain length, an observation also made by others [19].

Evidence that kasA encodes a Kas sensitive to cerulenin

The first step of chain elongation in fatty acid biosynthesis is the condensation of an acyl group, bound to ACP, with malonyl-ACP catalysed by a Kas. To determine directly the nature of the enzyme activity encoded by *kasA*, we have produced the key recombinant proteins, holo-AcpM, C_{16} -AcpM, mtFabD and KasA, in *E. coli* to develop an *in vitro* elongation–condensation assay. We were able to obtain the pure enzyme by loading the clarified soluble lysate on to a Ni^{2+} -charged His-Trap column. KasA was obtained as a pure protein migrating at approx.

Figure 3 FAS-II enzymic assay with *M. smegmatis* overexpressing *M. tuberculosis* KasA

The FAS-II assay was performed using different primers (C_2 – C_{16} -CoA) and the enriched soluble-cytosolic fraction (40–80%-saturated ammonium sulphate fraction) from *M. smegmatis* harbouring either the control plasmid pMV261 or pMV261::kasA-MTB. The inset shows the chain length distribution of FAMES synthesized from C_{12} -CoA and C_{14} -CoA in the FAS-II assay. Radiolabelled FAMES (7000 c.p.m.) were loaded on to C_{18} reverse-phase TLC in each lane, developed in $CHCl_3/CH_3OH$ (2:3, v/v), and the products were revealed by exposure to Kodak X-Omat film after a period of 4 days. Methyl $[1-^{14}C]$ palmitate was used as a reference standard.

with C_{16} -CoA and C_{16} -AcpM. When C_{16} -CoA was incorporated (in equimolar concentrations of C_{16} -AcpM) into the condensation assay, the product formation was reduced dramatically (see Figure 4A) to approx. 4 % of that observed with C_{16} -AcpM. This is consistent with our current understanding of mycobacterial FAS-II and the notion that KasA is a Kas involved in mero-mycolate extension, which utilizes acyl-AcpM-bound precursors.

We have shown previously that cerulenin exhibits potent *in vivo* anti-mycobacterial action [10]. Although overexpression of KasA in *M. bovis* BCG did not generate an increased resistance against cerulenin, it was suggested that the *in vivo* anti-mycobacterial activity of cerulenin was primarily via inhibition of earlier events catalysed by *de novo* FAS-I, thus masking inhibition of FAS-II. In this regard, we report in the present study an *in vitro* IC_{50} value of 20 $\mu g/ml$ (results not shown), for crude cytosolic *M. smegmatis* FAS-I preparations. Earlier studies have shown that cerulenin is a potent inhibitor of both FAS-I and FAS-II systems [20]. In addition, the *in vitro* inhibition of the *E. coli* Kas, FabB and FabF, has also been well documented for cerulenin [4]. It was thus reasonable to assume that cerulenin may also inhibit the β -ketoacyl-AcpM synthase activity of KasA. Increasing concentrations of cerulenin were added to the *in vitro* condensation assay. As shown in Figure 4(B), a marked dose-response inhibition of KasA activity was observed with cerulenin providing an IC_{50} value of 0.15 $\mu g/ml$ (0.67 μM). The IC_{50} values of the purified *E. coli* Kas proteins were reported as 3 and 20 μM for FabB and FabF respectively [21], suggesting that the mycobacterial condensing enzyme is more susceptible to cerulenin inhibition *in vitro* than the related *E. coli* enzymes.

Elongation activity of the KasA mutants

Siggaard-Anderson has identified a number of conserved amino acid residues in the *E. coli* FabB protein [22]. For instance,

Figure 4 KasA-mediated *in vitro* condensation activity

(A) KasA and its various mutants were expressed as His-tagged recombinant proteins in *E. coli* C41(DE3) and purified as described in the Materials and methods section. The purity of the recombinant proteins was assessed using SDS/PAGE [10% (w/v) gel] and the proteins visualized by staining with Coomassie Blue (inset). Each protein was assayed for Kas activity as described in the Materials and methods section. The condensation rates for KasA were tested with equimolar amounts of either C_{16} -AcpM or C_{16} -CoA as acyl sources. The activity of each of the mutants was assayed using C_{16} -AcpM and compared with KasA. (B) The condensation rates for KasA using C_{16} -AcpM as a substrate were determined as described above in the presence of increasing concentrations of cerulenin [CER].

Cys¹⁶³ constitutes the active-site residue. Other residues, such as His²⁹⁸, Lys³²⁸ and His³³³ were also identified and when replaced by Ala, decarboxylation and the overall elongation activity were abolished completely, suggesting the importance of these amino acids in catalysis of Kas-related proteins. The alignment presented in Figure 2(B) shows that these residues are also highly conserved in the KasA and KasB proteins, in both *M. smegmatis* and *M. tuberculosis*. In addition, we recently presented a structural model of the *M. tuberculosis* KasA generated by computing analysis based on the determined X-ray structure of the *E. coli* FabF protein by Huang et al. [23]. The three-dimensional structural model showed Cys¹⁷¹, His³¹¹, Lys³⁴⁰ and

His³⁴⁵ as part of the active site of KasA [10]. Site-directed mutagenesis was used to determine the role of these four residues in KasA activity by replacing them individually by alanine. The mutated KasA proteins were purified and CD-spectral analysis was performed, which is comparable with wild-type KasA (results not shown). KasA activity of all the mutant proteins (mutated at Cys¹⁷¹, His³¹¹, Lys³⁴⁰ and His³⁴⁵ to Ala) was abolished (Figure 4A), confirming the importance of these amino acids in KasA activity.

DISCUSSION

Kas of the type II system belongs to an important family of condensing enzymes that are related both structurally and functionally. They play key roles in the biosynthesis of fatty acids, polyketides and mycolic acids in mycobacteria [10,24]. In *E. coli*, KasIII (FabH) catalyses the elongation of an acetyl-CoA primer by malonyl-ACP [4,25]. In contrast, KasI (FabB) and KasII (FabF) only utilize acyl-ACP primers for elongation by malonyl-ACP [4,26]. Analysis of the *M. tuberculosis* genome database shows that there are only three potential Kas enzymes [24]. We have shown previously that mtFabH from *M. tuberculosis* corresponds to the *E. coli* KasIII enzyme (FabH), although it shows high-substrate specificity for C₁₂-CoA and C₁₄-CoA but not acetyl-CoA, and has been proposed as the critical enzyme that produces the primers following a complete cycle of elongation catalysed by FAS-II, presumably for KasA [7].

Mdluli et al. [6] have shown that isoniazid (INH) treatment of *M. tuberculosis* inhibits mycolic acid synthesis and is accompanied by a marked up-regulation of both AcpM and KasA, which are linked genetically. They also proposed KasA to be an INH target in *M. tuberculosis*, on the basis of the detection of an interaction between INH and AcpM. A recent study [10] suggests that KasA belongs to FAS-II and participates in mycolic acid biosynthesis. Therefore due to the importance of the FAS-II elongation process and the possible participation of KasA in relation to INH inhibition [6], we have initiated a number of studies to determine the role of KasA in mycolic acid biosynthesis. First, we examined the *in vivo* influence of KasA on mycolate production (α versus α'). Secondly, we analysed *in vitro* its overall effect on FAS-II activity within an enriched cytosolic protein fraction. Furthermore, we have defined its precise enzymic activity in a classical condensation assay using highly purified recombinant KasA, as well as its sensitivity to cerulenin, a well-known Kas inhibitor [4].

Overexpression of KasA from *M. tuberculosis* was associated *in vivo* with a decrease in α' -mycolate production and a concomitant increase in α -mycolates, both in *M. smegmatis* and in *M. chelonae*, suggesting that KasA is able to elongate the short α' -chain into full-length meromycolates. To investigate whether the presence of α' -mycolates in *M. smegmatis* was due to either the absence of the *kasA*-coding sequence, or the presence of an enzyme probably with reduced elongation activity, via aberrant mutations, we subsequently identified, cloned and expressed *kasA* from *M. smegmatis*. Overproduction of KasA_{MSG} in *M. smegmatis* resulted in similar changes in the α/α' ratio. Therefore the presence of α' -mycolates in some mycobacterial species cannot be inferred by the absence of *kasA*, or by an alteration of KasA activity via point mutations, since it was very similar to KasA-MTB. The α' composition may be simply due to different regulation levels of *kasA* expression and/or of other genes involved in the mycolic acid biosynthetic pathway in *M. smegmatis*. Furthermore, by analysis of the FAS-II activity of the cytosolic fraction of *M. smegmatis* using a set of acyl primers ranging from C₂- to C₂₀-CoA, we observed (following transacylation to AcpM)

that FAS-II activity was optimal with C₁₄/C₁₆-acyl-CoA/AcpM precursors. Interestingly, these substrates are the major products produced by mtFabH [7]. By comparing the FAS-II activity with a recombinant strain overproducing KasA, we found that although the same substrate preference was followed, the specific activity was enhanced significantly. This suggests that KasA is part of the mycobacterial FAS-II system.

Although KasA was produced in large amounts in different expression systems, it was always found to be highly insoluble (results not shown). We describe an expression system and culture conditions that allow the generation of soluble KasA in the present study. Recombinant KasA purified under non-denaturing conditions was assayed and found to catalyse the transfer of a two-carbon unit from [¹⁴C]malonyl-ACP to C₁₆-AcpM, thus confirming KasA as a condensing enzyme involved in FAS-II and meromycolate extension. To improve our understanding of the catalytic site of KasA, Cys¹⁷¹, His³¹¹, Lys³⁴⁰ and His³⁴⁵ were mutated. All mutations abrogated the enzymic activity, suggesting that these residues play a critical role in condensation activity mediated by KasA. Mutations in KasA (mutated at Asp⁶⁶ to Asn, Gly²⁶⁹ to Ser, Gly³¹² to Ser and Phe⁴¹³ to Leu), which also correlate with low levels of INH resistance, are supposed to inhibit the formation of a trimolecular complex consisting of KasA, INH and AcpM [6]. Interestingly, these mutations do not reside within the catalytic site [10], suggesting that, when compared with INH susceptibility/resistance, these mutations may rather influence the degree of binding of the acyl-AcpM or the stability of the KasA dimer. Clearly, the generation of substrates, such as C₁₆-AcpM and purified KasA, now allows the possibility to study key interactions between KasA and long-chain acyl-ACPs, in addition to how these mutations affect the KasA–INH–AcpM complex [10].

The type II Kas enzymes are commonly separated into two classes based on their sensitivity to cerulenin. In *E. coli*, cerulenin binds irreversibly to Kas proteins [4]. The mechanism of inhibition involves covalent cross-linking of the Kas active-site cysteine to cerulenin. The crystal structures of the FabB and FabF active sites [21,23,27,28] show that a hydrophobic binding pocket is present and occupied by the inhibitor. Structural analyses of Kas–cerulenin complexes suggest that cerulenin mimics the transition state of the condensation reaction effectively [21]. Cerulenin-sensitive Kas enzymes have a catalytic triad, which in FabB consists of Cys¹⁶³, His²⁹⁸ and His³³³ [21]. Protein alignments among FabB, FabF and KasA reveal a full conservation of these three residues [10], which may explain the sensitivity of KasA to cerulenin. In contrast, mtFabH, another Kas, has been shown to be resistant to cerulenin [7]. Altogether, this work and the recent studies by Schaeffer et al. [29] contribute to the understanding of the condensation–reaction mechanism mediated by KasA leading to mycolic acids via the cascade of FAS-II and now allows us to explore the development of new anti-mycobacterial agents that target this key condensation step in *M. tuberculosis*.

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