MabA (FabG1), a *Mycobacterium tuberculosis* protein involved in the long-chain fatty acid elongation system FAS-II

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The fatty acid elongation system FAS-II is involved in the biosynthesis of mycolic acids, which are very long-chain fatty acids of the cell envelope specific to *Mycobacterium tuberculosis* and other mycobacteria. A potential component of FAS-II, the protein MabA (FabG1), was overexpressed and purified. Sedimentation equilibrium analyses revealed that MabA undergoes a dimer to tetramer self-association with a dissociation constant of 22 μ M. The protein was detected by Western blotting in a mycobacterial cell-wall extract that produces mycolic acids and in the FPLC FAS-II fraction. MabA was shown to catalyse the NADPH-specific reduction of β -ketoacyl derivatives, equivalent to the second step of a FAS-II elongation round. Unlike the known homologous proteins, MabA preferentially metabolizes long-chain substrates (C_8 – C_{20}) and has a poor affinity for the C_4 substrate, in agreement with FAS-II specificities. Molecular modelling of MabA structure suggested the presence of an unusually hydrophobic substrate-binding pocket holding a unique Trp residue, suitable for fluorescence spectroscopic analyses. In agreement with the enzyme kinetic data, the spectral properties of MabA were different in the presence of the C_8 – C_{16} ligands as compared to the C_4 ligand. Altogether, these data bring out distinctive enzymic and structural properties of MabA, which correlate with its predilection for long-chain substrates, in contrast to most of the other known ketoacyl reductases.

Keywords: β -ketoacyl reductase, quaternary structure, enzymic activity, structural model, fluorescence spectroscopy

INTRODUCTION

Mycolic acids, very long-chain fatty acids (C_{60} – C_{90}), play an essential role in the architecture and permeability of the envelope of mycobacteria, including *Mycobacterium tuberculosis* (Liu *et al.*, 1996; Daffé & Draper, 1998). The front-line antituberculous drug isoniazid

(INH) impairs biosynthesis of these α -branched and β -hydroxylated molecules by inhibiting the production, by the fatty acid elongation system called FAS-II, of medium- to long-chain fatty acids, which would be precursors of mycolic acids (Takayama *et al.*, 1972; Quémard *et al.*, 1991; Marrakchi *et al.*, 2000; Vilchèze *et al.*, 2000). Indeed, INH inhibits a 2-*trans*-enoyl-acyl carrier protein (ACP) reductase (ENR), called InhA (Quémard *et al.*, 1996; Zabinski & Blanchard, 1997; Rozwarski *et al.*, 1998), that catalyses the last step of the four-step elongation rounds performed by FAS-II (Marrakchi *et al.*, 2000). InhA has been shown to be an essential enzyme for mycobacterial viability (Vilchèze *et al.*, 2000), leading to the conclusion that the production of long-chain fatty acids and mycolic acids is essential for mycobacterial growth. It has been suggested that the

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Abbreviations: ACP, acyl carrier protein; ESI, electrospray ionization; FAS, fatty acid synthetase; H-MabA, His-tagged MabA; INH, isoniazid; MALDI-TOF, matrix-associated laser desorption ionization-time of flight; KAR, β -ketoacyl-ACP reductase; PDB, Protein Data Bank; SDR, short-chain dehydrogenases/reductases.

KasA protein, which catalyses the first step of FAS-II elongation rounds, would also be a target of INH (Mdluli *et al.*, 1998). However, it has recently been shown that overexpression of the *kasA* gene did not modify the INH-susceptibility level of the vaccine strain *M. bovis* BCG, and mutations found in the *kasA* gene of INH-resistant clinical isolates are also present in INH-susceptible strains (Kremer *et al.*, 2000).

The FAS-II system has been isolated from a non-pathogenic mycobacterium, *M. smegmatis* (Odrizola *et al.*, 1977). It is a complex of several monofunctional enzymes that catalyses the ACP-dependent elongation of palmitoyl-CoA (C_{16}) into C_{18} – C_{30} saturated fatty acids, using malonyl-CoA as an elongation unit (Bloch, 1977). The mycobacterial FAS-II is an unusual type II system in that it elongates medium-chain-length substrates, while the other known bacterial systems perform *de novo* biosynthesis (Bloch, 1977). Only some of the proteins forming FAS-II have been positively identified, namely ACP (Bloch, 1977, Kremer *et al.*, 2001), and more recently InhA, KasA and mtFabD (Marrakchi *et al.*, 2000; Kremer *et al.*, 2000, 2001). The *inhA* gene is assumed to form an operon together with the upstream ORF, *mabA* (*fabG1*) (Banerjee *et al.*, 1994). The latter encodes a protein whose predicted amino acid sequence displays similarities with β -ketoacyl-ACP reductases (KARs or FabG), and a total soluble protein extract of *Escherichia coli* expressing *mabA* had KAR activity (Banerjee *et al.*, 1998). Cloning, overexpression and purification were performed in order to obtain the pure MabA protein for both biochemical and biophysical characterizations. Here we report enzymological and structural analyses of MabA that represent a fundamental step towards the design of inhibitors. Specific structural features of MabA, interrelated with its substrate specificity, could allow the development of new antibiotics directed against mycobacteria.

METHODS

Cloning and overexpression of *mabA*. The *mabA* (*fabG1*, Rv1483) gene of *M. tuberculosis* H37Rv was amplified by PCR from the cosmid MTCY277 (provided by Dr. S. Cole, Institut Pasteur, Paris, France) using *Pfu* DNA polymerase (Stratagene) and primers which contained *NdeI* and *XhoI* restriction sites, respectively (5'-GTCCATATGACTGCCA-CAGCCACTG-3', forward primer, and 5'-GTCTCGAGCG-TCTTGTTGTGTC-3', reverse primer; restriction sites are underlined). PCR was performed for 10 cycles, in the presence of 5% (v/v) formamide and 10% (v/v) DMSO, at an annealing temperature of 60 °C. The PCR product was ligated with T4 DNA ligase (Promega) downstream of a His-Tag oligohistidine-encoding sequence into the pET-15b overexpression vector (Novagen), which had been previously treated with *NdeI* and *XhoI* restriction enzymes. This construct was used to transform *E. coli* BL21(DE3) (Novagen). The absence of mutation was verified by sequencing the cloned *mabA* gene. The recombinant strain was grown at 37 °C in a rotary shaker (250 r.p.m.) in 200 ml Luria broth supplemented with 50 µg carbenicillin ml⁻¹. Expression of *mabA* was induced by the addition of 0.8 mM IPTG at an OD₆₀₀ of 0.8–0.9, and a further 2 h incubation at 37 °C.

Purification of MabA protein. Induced recombinant bacteria were washed with cold 50 mM potassium phosphate buffer pH 7.8 (buffer A), and resuspended in 4 ml of the same buffer supplemented with 500 mM NaCl and 5 mM imidazole. Cells were broken by one freezing/thawing cycle at –70 °C, in the presence of protease inhibitors [aprotinin, soybean trypsin-inhibitor, TLCK (*N*-*p*-tosyl-lysine chloromethyl ketone), pepstatin, leupeptin and PMSF] and 0.5 mg lysozyme ml⁻¹. Nucleic acids were removed by DNase I (5 µg ml⁻¹) and RNase A (10 µg ml⁻¹) treatments in the presence of MgCl₂ (10 mM) at 4 °C for 15 min. After centrifugation at 44 000 *g* for 15 min, the supernatant supplemented with 10% (v/v) glycerol was applied to a Ni-NTA Agarose (Qiagen) column (0.5 ml bed volume). After extensive washes by 5 mM then 50 mM imidazole in buffer A supplemented with 500 mM NaCl, MabA was eluted with 175 mM imidazole in buffer A supplemented with 500 mM NaCl. Fractions containing the His-tagged MabA (H-MabA) were identified by SDS-PAGE, pooled, dialysed twice against 50 vols 50% (v/v) glycerol in 50 mM potassium phosphate buffer, pH 7.2, and stored at –20 °C.

The InhA protein from *M. tuberculosis* was overproduced in *E. coli* and purified as previously described (Quémard *et al.*, 1995).

Mass spectrometry (MS). Electrospray ionization (ESI) analysis of MabA was performed using a TSQ 700 (Finnigan MAT) quadrupole mass spectrometer. The protein was dissolved in methanol/water/acetic acid (50:49.5:0.5, by vol.) and introduced by a syringe pump (Harvard) at a flow rate of 5 µl min⁻¹ into the electrospray source (5 kV, 250 °C). Nitrogen (pressure 40 p.s.i., 276 kPa) was used as nebulizing gas. MALDI-TOF spectra (in the positive mode) were acquired on a Voyager-DE STR Biospectrometry workstation (PerSeptive Biosystems), equipped with a pulsed nitrogen laser emitting at 337 nm, as described by Laval *et al.* (2001), using an extraction voltage of 20 kV and 2,5-dihydroxybenzoic acid solution [10 mg ml⁻¹ in water/acetonitrile, 8:2 (v/v)] as matrix.

Analytical size-exclusion chromatography. A prepacked Sephacryl S-100HR column (Amersham Pharmacia Biotech), monitored by a BioCAD SPRINT system (PerSeptive Biosystems), was equilibrated in 50 mM potassium phosphate buffer, pH 6.8, containing 100 mM NaCl. H-MabA (0.7 mg) and InhA (1.1 mg) were separately eluted through the column with the same buffer, at a flow rate of 0.5 ml min⁻¹. The column was calibrated in the same elution conditions by applying, in two separate elutions, the following standard proteins (0.5–1 mg of each protein): alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and RNase A (13.7 kDa). The molecular mass of H-MabA was estimated from the calibration curve of elution volumes versus molecular masses.

Analytical ultracentrifugation. Analytical ultracentrifugation experiments were performed in a Beckman Optima XL-I analytical ultracentrifuge using an An-55 four-hole rotor with six-channel Epon charcoal-filled centrepieces, and absorbance optics. Prior to the measurements, the protein samples were eluted through a Fast desalting column HR 10/10 (Amersham Pharmacia Biotech) in 20 mM MES, pH 6.4, 300 mM LiSO₄ and 1 mM tris-(carboxyethyl)phosphine hydrochloride. Sedimentation equilibrium experiments were carried out at 4 °C on sample volumes of 110 µl, at loading MabA concentrations of 26, 39 and 78 µM, and at rotor speeds of 4872, 7015 and 14637 *g* (respectively 7500, 9000 and 13 000 r.p.m.). Data were

collected at 280 nm and runs were continued until there was no significant difference in scans taken 2 h apart. Data were analysed with the XL-A/XL-I Data Analysis software version 4.0 (Beckman). The partial specific volume (0.7223 ml g^{-1}) of the recombinant H-MabA protein and the solvent density (1.04 g ml^{-1}), at the experimental temperature, were calculated with help of the program Sednterp (Sedimentation Interpretation Program, version 1.05).

Preparation of FAS-I and FAS-II systems or cell-wall extract, and Western blots. Cell-wall extract, FAS-I and FAS-II systems were prepared from *M. smegmatis* mc²155 as previously described (Marrakchi *et al.*, 2000). An aliquot of total protein extract was kept after sonication of bacteria, during preparation of FAS-I and FAS-II systems. Samples were separated by 12% or 15% (w/v) polyacrylamide SDS-PAGE, and proteins transferred onto a nitrocellulose membrane. Polyclonal rabbit antibodies against pure H-MabA protein were used for Western blot analyses. Rabbit preimmune serum was used for control experiments. Antigen-antibody interactions were revealed by colorimetric reaction, using alkaline phosphatase-mouse anti-rabbit IgG conjugates.

Synthesis of different chain length β -ketoacyl-CoAs. β -Ketoacyl-CoAs possessing 8–20 carbon atoms were synthesized as described by Vagelos & Alberts (1960), with some modifications. For solubility reasons, for the C₁₆ and C₂₀ derivatives the deprotection step of the ketone function was realized by a transacetalization reaction, by incubating the ethylenic ketal derivatives in anhydrous acetone and *p*-toluenesulphonic acid (10 mM) at 56 °C, for 36 h. The derivatization into acyl-CoAs was performed at pH 8.7, in 600 mM Tris/tetrahydrofuran (1:1, v/v). The compounds obtained at each synthesis step were purified and their structure characterized by electron-impact MS, IR and NMR spectroscopies. The final β -ketoacyl-CoA products were purified by chromatography on a C₁₈ Sep-Pak cartridge equilibrated with 20 mM NaH₂PO₄ and elution with a 0–80% methanol gradient in water, and their structures verified by ESI-MS in the negative mode.

Enzyme assays and steady-state kinetics. Kinetic parameters were determined spectrophotometrically by following NADPH oxidation at 340 nm using a thermostatted Uvikon 923 spectrophotometer (Kontron Instruments). Standard reactions were performed in a quartz cuvette in a total volume of 1 ml, at 25 °C, in 100 mM sodium phosphate buffer, pH 7.0, in the presence of fixed concentrations of NADPH and β -ketoacyl-CoA; after equilibration of the baseline, reactions were started by adding a defined amount of H-MabA enzyme, and measurements performed for 3–5 min. Comparison of the initial reaction rates obtained for the different chain length β -ketoacyl-CoAs at a low substrate concentration was performed at 2 μM β -ketoacyl-CoA, 100 μM NADPH and 18–360 nM H-MabA (depending on the substrate used). The steady-state K_m value for NADPH was determined by measuring initial velocities at various concentrations of coenzyme and at fixed concentrations of acetoacetyl-CoA (400 μM) and of enzyme (36 nM). The K_m values for β -ketoacyl-CoAs and k_{cat} values were measured by varying the concentration of each β -ketoacyl-CoA and at fixed concentrations of NADPH (100 μM) and of enzyme (18–144 nM, depending on the substrate). Data were fitted to the Michaelis-Menten equation by least-squares fits to a hyperbola using the program GraphPad Prism to calculate the kinetic parameters. For MALDI-TOF MS analyses, reactions were performed in a total volume of 1 ml, at 25 °C, in the presence of 300 μM NADPH, 200 μM acetoacetyl-CoA and 800 nM H-MabA, in

20 mM Tris buffer, pH 7.0, and stopped after completion (monitored by A₃₄₀ measurement).

Sequence comparison and molecular modelling. Protein sequence database searches were performed with PSI-BLAST version 2.0.5 (Altschul *et al.*, 1997), with default parameters. Alignment refinement was subsequently performed using the program TITO (Labesse & Moron, 1998) and structures of related enzymes (see text). MabA secondary structures (α -helices, β -strands) were assigned during TITO processing, and the secondary structures derived by homology were used as additional restraints in the following modelling step (except for the stretch 48-GSGAPKG-54 due to its sequence enriched in glycine). The three-dimensional model was built using the quaternary structure deduced from the crystal structure of PDB1EDO (40% identical) as a template in the program MODELLER 4.0 (Sali & Blundell, 1993) and assessed using Verify-3D (Eisenberg *et al.*, 1997) and PROSA (Sippl, 1993). These three-dimensional structures were visualized on a UNIX workstation using the program InsightII (MSI, San Diego, USA).

Fluorescence spectroscopy. Steady-state emission spectra were recorded on an ISS K2 spectrofluorimeter through a 8 nm bandwidth monochromator and corrected for buffer emission. Time-resolved data were collected in the frequency domain using ISS acquisition electronics and analysed with the software Globals Unlimited (Beechem *et al.*, 1991). The excitation light was at 300 nm from the frequency-tripled output of a pulse-picked Tsunami Ti-Sa laser pumped by a Millennia X dye laser (Spectra Physics). The reference lifetime compound was *N*-acetyltryptophanamide in water (3 ns). Measurements were carried out using 6 μM H-MabA in 100 mM sodium phosphate buffer, pH 7.0, and 7% (w/v) glycerol, in the presence or absence of a ligand (at saturating concentration of NADP⁺ and/or K_m concentration of β -ketoacyl-CoA). Fluorescence spectroscopy in the presence of NADPH was not performed as the coenzyme absorption spectrum overlaps the emission spectrum of the protein tryptophan residue.

RESULTS

Expression of the *M. tuberculosis* MabA protein in *E. coli* and purification

The *mabA* gene of *M. tuberculosis* was cloned into the plasmid pET-15b. The N-terminal His-tagged MabA protein (H-MabA) was produced at high levels (30% of the total soluble proteins) in *E. coli* BL21(DE3), and purified to greater than 96% purity by chromatography over a Ni-NTA agarose column (Fig. 1). After dialysis, the amount of purified protein recovered corresponded to the quasi-total amount of H-MabA loaded onto the column. ESI-MS analysis showed the mass of the protein to be 27729 Da, which is 131 Da below the predicted average mass for H-MabA (27860: 25697 + 2163). Amino acid sequencing revealed that this mass difference corresponds to the loss of the methionine at the N-terminus of the recombinant protein.

Quaternary structure of MabA

The quaternary structure of MabA was analysed by gel filtration. The chromatogram suggested the presence of two molecular species (a major peak at 95 kDa and a

Fig. 1. Expression of H-MabA protein in *E. coli* and purification. SDS-PAGE analysis of 25 µg of each sample is shown: (1) molecular mass markers, (2) crude extract of *E. coli* BL21(DE3)/*h-mabA* treated with IPTG, (3) total soluble protein extract of *E. coli* BL21(DE3)/*h-mabA* loaded onto the Ni-NTA agarose column, (4) first wash with 5 mM imidazole, (5) first wash with 50 mM imidazole, (6) pooled fractions after elution of H-MabA with 175 mM imidazole. The gel was stained with Coomassie blue.

Fig. 2. Equilibrium sedimentation of MabA. The upper part of the panel shows the residual difference between experimental and fitted values by its standard deviation. This experiment was carried out at three different centrifugation speeds and three different protein concentrations (see Methods), and was repeated twice using distinct protein batches. An identical fit was performed for all datasets, but only the result obtained with 78 µM MabA at 9000 r.p.m. is shown here for more clarity.

minor one at 58 kDa). The InhA protein (114 kDa), used as a reference, eluted as a single symmetrical peak estimated at 111 kDa, in agreement with a previous report (Rozwarski *et al.*, 1999). Sedimentation equilibrium analyses displayed an increase of the apparent weight-average molecular weight ($M_{w,app}$) with absorbance, indicating the behaviour of a self-associating system of several molecular species (data not shown). The maximum value of $M_{w,app}$ (around 100 kDa) suggested that the largest species was a tetramer (110.9 kDa). When datasets were analysed separately by non-linear least-squares fittings, best fits were obtained for the dimer–tetramer model (Fig. 2). The other models gave variances and 95% confidence intervals that were too large, systematic errors on the residuals plots or aberrant K_d values. Global simultaneous fittings of all datasets gave a dimer–tetramer equilibrium association constant of 7.9 ± 0.7 ml mg⁻¹, corresponding to a K_d of 22 ± 2 µM. Thus, gel filtration chromatography and sedimentation equilibrium experiments both showed that MabA can self-associate in a tetrameric structure, as observed for KARs from plants (Sheldon *et al.*, 1990, 1992; Fisher *et al.*, 2000).

Coelution of MabA with the FAS-II system

MabA was detected by Western blotting in a mycobacterial cell-wall extract (Fig. 3b) that produces mycolic acids and contains the FAS-II complex (Marrakchi *et al.*, 2000). The extra band observed may be linked to proteolysis of MabA or to cross-reactivity of the

Fig. 3. Detection of MabA in *M. smegmatis* protein fractions by Western blotting. (a) Analysis of FAS-I and FAS-II. (b) Analysis of cell-wall extract. Lanes: (1) pre-stained molecular standards, (2) pure H-MabA, (3) total sonication extract from *M. smegmatis*, (4) fraction FAS-I, (5) fraction FAS-II, (6) cell-wall extract. For SDS-PAGE, 25 µg of proteins of each sample and 10 ng of pure H-MabA were used. For Western blotting, polyclonal rabbit anti-MabA antibodies were used. No signal was obtained using the rabbit preimmune serum (data not shown).

antibodies with another KAR, since there are four other putative KARs in *M. tuberculosis*. The mycobacterial fatty acid biosynthesis systems FAS-I and FAS-II were

Fig. 4. Reaction scheme for the reduction of β -ketoacyl-CoA substrates by MabA.

Table 1. Kinetic parameters of the MabA protein

Reactions were run at 25 °C, in 100 mM sodium phosphate buffer, pH 7.0, and were followed spectrophotometrically at 340 nm (see Methods). Data were fitted by non-linear least-squares regression to calculate the kinetic parameters. Values are means of three separate experiments $\pm$ SD.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)
NADPH	41 ± 7	2.6 ± 0.2	0.063 ± 0.015
Acetoacetyl-CoA (C_4)	1530 ± 81	1.9 ± 0.0	0.001 ± 0.000
β -Ketooctanoyl-CoA (C_8)	70 ± 8	3.5 ± 0.2	0.050 ± 0.008
β -Ketododecanoyl-CoA (C_{12})	8.3 ± 0.8	4.3 ± 0.2	0.520 ± 0.074

isolated by FPLC, and Fig. 3(a) shows that MabA coeluted with FAS-II, but not with FAS-I.

Enzymic activity of the MabA protein

The reduction of β -ketoacyl derivatives by the recombinant purified protein (Fig. 4) was monitored by spectrophotometry. It showed that H-MabA is active in the simultaneous presence of acetoacetyl-CoA (C_4 substrate) and NADPH. MALDI-TOF MS analyses showed that the protein reduces acetoacetyl-CoA ($[M+H]^+$ at m/z 852, $[M+Na]^+$ at m/z 874) to hydroxybutyryl-CoA ($[M+H]^+$ at m/z 854, $[M+Na]^+$ at m/z 876). This activity was linearly dependent on the concentration of H-MabA, and was not detected when NADPH was replaced by NADH, indicating that the enzyme is NADPH-dependent, like most of the known KARs (Bloch, 1977). In the absence of NADPH, the production of saturated fatty acids by the FAS-II system was totally abolished, while β -ketoesters accumulated (data not shown), consistent with MabA being a key component of FAS-II.

Steady-state kinetic experiments (Table 1) gave a K_m value of MabA for acetoacetyl-CoA of 1530 μM , which is 6 times the K_m of KARs from plants (Sheldon *et al.*, 1992; Shimakata & Stumpf, 1982). In contrast, the k_{cat} and the K_m for NADPH reported for these proteins are of the same order of magnitude as those measured for MabA (Table 1).

To determine the substrate specificity of the protein, longer-chain β -ketoacyl-CoAs were synthesized. The measured kinetic parameters revealed a much better activity of MabA with long-chain substrates, since the K_m and the k_{cat} significantly improved with C_8 and C_{12}

derivatives (Table 1). The kinetic parameters for the C_{16} and C_{20} substrates could not be determined because of an inhibition phenomenon at high substrate concentration, which has been observed for other fatty acid biosynthesis systems (Bloch, 1977). Initial reaction rates were nevertheless compared at a very low substrate concentration (2 μM), in the presence of β -ketoacyl-CoAs ranging from C_8 to C_{20} . The highest values of initial velocity were obtained with the C_{12} and C_{16} substrates [V_i values of 0.7 ± 0.1 and $0.4 \pm 0.0 \mu\text{mol min}^{-1}$ (mg protein^{-1}), respectively]; moreover, the C_{20} derivative appeared to be a better substrate than the C_8 one (respectively 23 and 13 % of the V_i for C_{12}). These data showed that MabA has a predilection for long-chain substrates.

Structural analysis of the MabA protein

Through PSI-BLAST searches (Altschul *et al.*, 1997) performed in non-redundant databases, MabA appeared highly conserved among mycobacteria (81–84 % identity in *M. tuberculosis*, *M. avium* and *M. smegmatis*), while identity scores with other KARs (16 sequences) from various organisms, bacteria or plants, ranged from 29 to 43 % over the whole sequence. The search for related three-dimensional structures indicated extended similarities with the short-chain dehydrogenases/reductases (SDR) superfamily (Rafferty *et al.*, 1995; Jörnvall *et al.*, 1995), or the reductases/epimerases/dehydrogenases (RED) superfamily (Labesse *et al.*, 1994), to which InhA belongs, the closest structure being the KAR from *Brassica napus* (PDB1EDO), with 40 % identity. Most residues critical for cofactor binding and catalysis are well conserved in MabA sequence (Fig. 5). Furthermore, in agreement with the kinetic data, the $\beta 2$ strand displays

Fig. 5. Alignment of the MabA sequence with that of KARs from *B. napus*, *E. coli* and *Haemophilus influenzae*. The alignment was performed with CLUSTAL W (Thompson et al., 1994). Strictly conserved residues are highlighted with black boxes. Secondary-structure elements corresponding to MabA protein are drawn on the top of the alignment. The consensus sequences defining the SDR superfamily signature (Jörnvall et al., 1995; Labesse et al., 1994) are framed. Stars indicate the amino acids forming the catalytic triad; dotted lines indicate conserved motifs involved in the tetramer interfaces. The figure was drawn with the program ESPript (Gouet et al., 1999).

Fig. 6. Structural model of a MabA subunit complexed with one NADPH molecule. The trace of the amino acid chain is displayed as a ribbon. Secondary-structure elements are labelled. NADPH is drawn as blue sticks. (a) Complete view of the subunit. α -Helices are coloured in purple and β -strands in yellow. The cavity delimited by the C-termini of the β 4, β 5, β 6, β 7 strands and the α 4, α 5, α 6, α 6' and α 6'' helices correspond to the predicted substrate-binding site. (b) Top view of the predicted active site of MabA. A rotation of 90 degrees compared to the view in (a) was performed. The trace of the amino acid chain is coloured in purple. The side chains of selected amino acid residues are shown. Hydrophobic residues at the bottom of the substrate-binding pocket are coloured in green (Val¹⁴¹, Ile¹⁴⁷, Tyr¹⁸⁵, Ile¹⁹⁸, Gly²⁰¹, Ala²⁰², Phe²⁰⁵, Ile²⁰⁶, Val²¹¹, Met²⁴³), except for Trp¹⁴⁵, which is in yellow. The catalytic triad is coloured in red (Ser¹⁴⁰, Tyr¹⁵³, Lys¹⁵⁷), polar residues surrounding the triad are in white (Ser⁹², Ser¹⁴², Gln¹⁵⁰), and hydrophobic residues facing the triad are in orange (Met¹⁹⁰, Thr¹⁹¹, Leu¹⁹⁴). Panel (a) was produced using the MolScript program (Kraulis, 1991), and panel (b) using the InsightII program (MSI).

Fig. 7. Fluorescence emission of MabA in the presence of different ligands. Emission spectra in the absence of ligand (+) or in the presence of β -ketoacyl-CoAs C_4 (Δ), C_8 ($\blacktriangle$), C_{12} ($\bullet$), C_{16} ($\square$) or $NADP^+$ ($\blacksquare$). Excitation wavelength: 300 nm. AU, arbitrary unit. Similar data were obtained for the substrates in the presence of $NADP^+$.

a sequence motif (Fig. 5) typical of the NADP(H)-specific enzymes (Labesse *et al.*, 1994).

MabA protein structure was modelled with good scores [PROSA (Sippl, 1993), -1.50 ; Verify-3D (Eisenberg *et al.*, 1997), $+0.45$; versus -1.65 and $+0.55$, respectively, for the template] using the structure of PDB1EDO in complex with NADPH (Fisher *et al.*, 2000). The two mostly hydrophobic interfaces of dimerization (Fig. 5) found in the tetrameric SDR proteins (Rafferty *et al.*, 1995; Jörnvall *et al.*, 1995; Fisher *et al.*, 2000), are conserved in the MabA model. The nicotinamide portion of NADPH, involved in hydride transfer during catalysis (Fig. 4), points deep into the substrate-binding site of the protein (Fig. 6a). Three distinct parts may be described in this pocket (Fig. 6b). A patch of polar residues that surrounds the catalytic triad, and a mainly hydrophobic region facing the catalytic triad (Fig. 6b) are very well conserved among the known KARs (except

Ser¹⁴², which is often replaced by a residue of the same size, a Val) (Fig. 5). The bottom of the MabA substrate-binding pocket appeared mainly hydrophobic, and the unique tryptophan residue (Trp¹⁴⁵) was modelled in the vicinity of this cavity (Fig. 6b). Interestingly, in most other KARs, the hydrophobic residues Trp¹⁴⁵, Ile¹⁴⁷, Ile¹⁹⁸ and Phe²⁰⁵ observed in the MabA pocket are replaced by more polar residues (see Fig. 5). In contrast, these hydrophobic residues are perfectly conserved in MabA from *M. avium* and *M. smegmatis*. Furthermore, the very C-terminus [²⁴³MGMGH²⁴⁷] of the mycobacterial MabA proteins is highly specific to this subclass of KARs (data not shown). These specific structural features might correlate with the observed distinct substrate specificity. Comparison of our MabA model with the crystal structure of the ternary complex InhA–NAD⁺– C_{16} substrate (Rozwarski *et al.*, 1999) and manual fitting of the C_{16} substrate suggested that the MabA active site would accommodate the β -ketoacyl-CoA with minor rearrangements.

Fluorescence spectroscopy

The presence of a unique Trp residue in the MabA predicted substrate-binding pocket prompted us to test the effect of ligand binding on MabA fluorescence emission. Upon excitation at 300 nm, the emission spectrum displayed a maximum at about 340 nm (Fig. 7), which showed that the tryptophan is partially buried (Lakowicz, 1983). Upon addition of $NADP^+$, a diminution of fluorescence intensity and a blue-shifting of the λ_{max} (from 340 to 332 nm) were observed (Fig. 7). This fluorescence quenching arose from a decrease of the populations of the long- and middle-lifetime emitting species (Table 2), despite a slight increase of the lifetime values, showing a conformational restraint of the Trp residue dynamics. These phenomena, together with an increase of the fluorescence polarization (data not shown), showed that the substrate-binding pocket closes upon NADP binding. In the presence of acetoacetyl-CoA, a similar behaviour was observed by steady-state spectroscopy (Fig. 7) and differential anisotropy (data

Table 2. Time-resolved fluorescence of MabA

Analysis of the frequency response profiles of the MabA tryptophan fluorescence in the absence or presence of different ligands, β -ketoacyl-CoAs or $NADP^+$. Data were fitted using a triple exponential model and expressed as τ (fluorescence lifetime) values and as a percentage of emitting species for each lifetime (long, middle and short). These three lifetimes are expected for the three rotamers around the χ_1 angle of a Trp residue within a protein. In each case, the chi-square for the fit is less than 0.15. Measurements were repeated in two independent experiments, and representative results are shown.

Fluorescence lifetime	No ligand		C_4		C_8		C_{12}		C_{16}		$NADP^+$	
	τ (ns)	%	τ (ns)	%	τ (ns)	%	τ (ns)	%	τ (ns)	%	τ (ns)	%
Long	7.7	14	5.8	8	7.1	18	7.1	18	7.0	19	9.8	4
Middle	2.8	36	2.1	29	2.7	37	2.7	34	2.2	39	3.5	24
Short	0.5	50	0.3	63	0.5	45	0.5	47	0.4	41	0.5	72

not shown). In this case, however, the quenching arose from a decrease of all the three fluorescence lifetimes (Table 2), suggesting that the induced conformational change brings a quencher into the vicinity of the Trp residue.

Interestingly, replacement of acetoacetyl-CoA (C_4) by longer-chain substrates (C_8 – C_{16}), either in the presence or in the absence of $NADP^+$, induced a significant release of quenching (Fig. 7). The fractional populations and fluorescence lifetimes of MabA alone were mostly recovered (Table 2), suggesting that the Trp residue and the quencher do not interact as efficiently as in the presence of the C_4 substrate.

Thus, the fluorescence data correlate with the measured kinetic parameters since they both show a major difference between the C_4 and the longer substrates (Fig. 7, Tables 1 and 2).

DISCUSSION

The *mabA*–*inhA* operon on the *M. tuberculosis* chromosome encodes two biochemically and structurally related enzymes. This study provides evidence that the MabA protein, like InhA, belongs to the mycobacterial FAS-II system, and that MabA catalyses the $NADPH$ -dependent reduction of β -ketoacyl derivatives, which corresponds to the second step of the elongation round. Thus, MabA and InhA carry out the two reduction steps of the elongation pathway. The inferred MabA model suggests the conservation of the quaternary structure of SDRs, in agreement with experimental data. Furthermore, the two regions of the substrate-binding pocket surrounding and facing the catalytic triad are very well conserved when compared to other KARs. Similar environments have been found in two related $NADPH$ -specific keto reductases: the trihydroxynaphthalene reductase from *Magnaporthe grisea* (ThnR, PDB1YBV) and the tropinone reductase II from *Datura stramonium* (TR-II, PDB2AE2) (Andersson *et al.*, 1996; Nakajima *et al.*, 1999). These data lead us to propose similar orientations of the substrate keto function and catalytic mechanisms for the KARs (including MabA), ThnR and TR-II (Jörnvall *et al.*, 1995; Thompson *et al.*, 1997).

Both MabA and InhA have a large substrate-binding pocket, adequate to accommodate the long acyl chains elongated by FAS-II. The substrate specificity of MabA is also consistent with that of the *M. tuberculosis* KasIII (or mtFabH) protein, believed to play the role of pivotal link between FAS-I and FAS-II systems (Choi *et al.*, 2000). This specificity might partly reside in the relatively strong hydrophobicity of the MabA substrate-binding pocket, particularly in the bottom part, which appears globally more hydrophobic than that of the other known KARs. It is noteworthy that, in the crystal structure of the ternary complex InhA– NAD^+ – C_{16} substrate, numerous hydrophobic residues interact with the long aliphatic chain of the substrate (Rozwarski *et al.*, 1999). The steric hindrance and local conformational changes induced by a long acyl chain interacting with

residues lining the MabA substrate-binding pocket may explain the difference in fluorescence behaviour between the C_4 and C_8 – C_{16} substrates. Likewise, local rearrangements of the substrate-binding pocket of InhA and ThnR were observed upon binding of the substrate or an inhibitor (Rozwarski *et al.*, 1999; Andersson *et al.*, 1996).

The outbreak of resistant *M. tuberculosis* strains over the last 15 years has focused international attention on the need for new drug development. The structural and functional features of the MabA protein described here, especially its affinity for long-chain substrates, should allow the development of MabA-specific substrate analogues, as exemplified by thymidine monophosphate kinase of *M. tuberculosis* (Munier-Lehmann *et al.*, 2001), which might lead to the design of novel antituberculous drugs.

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