Evidence for specific and non-covalent binding of lipids to natural and recombinant *Mycobacterium bovis* BCG Hsp60 proteins, and to the *Escherichia coli* homologue GroEL

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Heat-shock proteins (Hsps) from various origins are known to share a conserved structure and are assumed to be key partners in the biogenesis of proteins. Fractionation of the mycobacterial Hsp60, a 65 kDa protein also called Cpn60, from *Mycobacterium bovis* BCG zinc-deficient culture filtrate on phenyl-Sepharose followed by Western blotting revealed the existence of four Hsp60-1 and Hsp60-2 forms, based on their hydrophobicity behaviour. Hsp60-2 species were further purified by ion-exchange chromatography and partial amino acid sequences of cyanogen bromide (CNBr) peptides of purified Hsp60-2 species showed identity with the amino acid sequence deduced from the *hsp60-2* gene, indicating that the various Hsp60-2 forms are encoded by the same gene. In addition, the mycobacterial Hsp60-2 was overexpressed in *E. coli* using the pRR3Hsp60-2 plasmid and analysed on phenyl-Sepharose. The elution pattern of the recombinant Hsp60-2, as well as that of *Escherichia coli* GroEL, was similar to that of the native Hsp60-2 from the culture filtrate of *M. bovis* BCG and entirely different from that of the mycobacterial antigen 85. Extraction of mycobacterial Hsp60-2 forms, recombinant BCG Hsp60-2 and *E. coli* GroEL with organic solvents releases various amounts of non-covalently bound lipids. The presence of lipids on Hsp60-2 was confirmed by labelling *M. bovis* BCG with radioactive palmitate. The radioactivity was specifically associated with Hsp60 in the aqueous phase and the 19 and 38 kDa lipoproteins in the Triton X-114 phase. Analysis of the lipids extracted from purified Hsp60-2, recombinant BCG Hsp60-2 and *E. coli* GroEL by TLC showed the same pattern for all the samples. Acid methanolysis of the lipids followed by GC analysis led to the identification of C_{16:0}, C_{18:0} and C_{18:1} as the major fatty acyl constituents, and of methylglycoside in these proteins. Altogether, these data demonstrate that lipids are non-covalently bound to Hsp60-2 and homologous proteins.

Keywords: *Mycobacterium bovis* BCG, Hsp60 protein, GroEL

INTRODUCTION

Three major classes of heat-shock protein (Hsp) are thought to be involved in protein biogenesis: Hsp60, Hsp70 and Hsp90. An important role of chaperones *in*

vivo is to prevent protein aggregation (Netzer & Hartl, 1998). Most Hsp60 proteins share a common oligomeric structure consisting of two stacked rings of seven subunits each. The most studied Hsp60 is the one from *Escherichia coli* named GroEL, whose crystal structure has been recently determined (Braig *et al.*, 1994). Mutations in different domains suggest that the association of essential functions, such as polypeptide

Abbreviations: CNBr, cyanogen bromide; Hsp, heat-shock protein; PB, phosphate buffer.

chain fixation site and ATP hydrolysis are located in precise regions of the protein (Fenton *et al.*, 1994).

The co-operating factor GroES (Hsp10) binds asymmetrically to GroEL, and stimulates ATP hydrolysis and substrate release (Hartl, 1996). Cell survival under stress conditions depends on the speed and efficiency of the activating mechanism responsible for the transcription of the *hsp* genes (Liberek & Georgopoulos, 1993; Morimoto, 1993) as well as on the ability of these Hsp proteins to prevent protein denaturation (Ziemienowicz *et al.*, 1993; Martin *et al.*, 1988). The Hsp proteins are highly conserved in nature from bacteria to humans (Harboe & Quayle, 1991; Thole *et al.*, 1988a; Thole & Van Der Zee, 1990). The immunological activities of Hsp60 in infectious diseases (Harboe & Quayle, 1991; Kaufmann, 1990; Welch & Winfield, 1992), in autoimmune reactions (Van Eden *et al.*, 1989; Young, 1992), in cancer (Jäättelä & Wissing, 1992) as well as their use as potential antitumour vaccines (Ahsan & Sasaki, 1991, 1993) are active fields of investigation.

Large amounts of Hsp60-2 (P64; GroEL-2; 65 kDa) can be obtained from culture filtrates of *Mycobacterium bovis* BCG grown on zinc-deficient Sauton medium (De Bruyn *et al.*, 1987a). Further systematic analysis by gel electrophoresis of the different fractions obtained during stepwise elution with buffers of decreasing ionic strength from hydrophobic phenyl-Sepharose, performed as an initial phase of purification, showed the presence of proteins exhibiting an apparent molecular mass of 65 kDa in all the eluted fractions, suggesting the existence of several Hsp60s differing by their hydrophobicity. The new species, with respect to their order of elution, were named Hsp60-2a, Hsp60-2b and Hsp60-2d in reference to the protein we previously characterized, which we renamed Hsp60-2c.

To find an explanation for the different hydrophobic behaviour of Hsp60 proteins, the present study was undertaken by investigating the possible association of lipids with various Hsp60s, namely Hsp60-2 (65 kDa) from *M. bovis* BCG, the recombinant Hsp60-2 protein produced in *E. coli* and, as an external reference, *E. coli* Hsp60 (GroEL).

METHODS

Bacterial strains and plasmids. *E. coli* LE392 (Promega) was used for cloning procedures. Bluescribe M13+ vector was purchased from Stratagene. pRIB1000 (Thole *et al.*, 1987), a pPLc236 derivative carrying a 4.9 kb *hsp60*-containing *M. bovis* BCG DNA fragment was a gift from J. Van Embden (National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands); pRR3 (Ranes *et al.*, 1990), derived from *M. fortuitum* pAL5000, was kindly provided by B. Gicquel (Institut Pasteur, Paris, France).

Construction of pRR3Hsp60-2. A 3 kb *Sma*I fragment of the plasmid pRIB1000 containing the promoter and the coding regions of *hsp60-2* was subcloned into a Bluescribe M13+ vector designated Bshsp. To construct the *hsp* expression vector under the control of its own promoter, Bshsp was

digested by *Sma*I and the resulting 3 kb fragment was ligated to *Sca*I-digested pRR3, a kanamycin-resistant mycobacteria/*E. coli* shuttle vector.

Reagents. Restriction enzymes, T4 DNA ligase and other DNA modifying enzymes were purchased from Boehringer Mannheim, Promega or USB.

Culture of bacteria. *M. bovis* BCG 1173P2 (Pasteur Institute, Paris, France) was grown as a pellicle at 37.5 °C, on normal or zinc-deficient Sauton medium. The medium was prepared with Milli RO water (conductivity 20 S); zinc sulfate was added to a final concentration of 5 µM (normal Sauton medium) or 0.15 µM (zinc-deficient medium). *E. coli* was grown on LB medium containing 25 µg kanamycin ml⁻¹ and harvested at an OD₆₆₀ between 0.6 and 0.8.

Filtrate from zinc-deficient culture. The culture medium was clarified by decantation. The remaining organisms were removed by filtration through a Pellicon filter unit of 0.22 porosity (Millipore). The culture filtrate was dialysed against 20 mM phosphate buffer (PB) pH 7.3 using a Pellicon filter unit equipped with membranes of 10 kDa cut off.

Preparation of cell extracts. *M. bovis* BCG extracts from 7-d-old cultures were prepared as previously described (De Bruyn *et al.*, 1987a). Briefly, the bacterial pellets were suspended in 0.05 M PB, pH 7.3 (0.3 g wet weight cells per ml buffer). The suspension was homogenized with a potter homogenizer, disrupted at 83–110 MPa in a French press and clarified by centrifugation at 4000 g. After a second centrifugation at 100 000 g for 90 min, the resulting supernatant was analysed by hydrophobic chromatography.

E. coli was harvested at an OD₆₆₀ between 0.6 and 0.8, centrifuged at 10 000 g for 30 min and washed twice with 0.05 M PB pH 7.3. The bacterial pellet was resuspended in the same buffer using the same ratio of wet weight cells per ml buffer as described for *M. bovis* BCG. The bacterial suspension was disrupted at a maximum of 83 MPa in a French press and clarified by centrifugation under the same conditions as *M. bovis* BCG.

Protein determination. Protein concentrations were determined with the Bio-Rad protein assay kit, according to the Bradford dye (Coomassie brilliant blue G)-binding procedure (Spector, 1978) with BSA as the standard. The amounts of non-covalently bound lipids on Hsp60 were estimated by dot blotting. Protein concentration was determined by the Lowry method.

Protein analysis by PAGE. SDS-PAGE was conducted as described by Laemmli (1970) on 13% (w/v) acrylamide gels. Proteins from zinc-deficient culture medium and from fractions eluted from the first purification step on phenyl-Sepharose were precipitated by 10% (w/v) trichloroacetic acid in the presence of sodium deoxycholate (Sigma) at a concentration of 125 µg ml⁻¹ and kept on ice for 2 h (Bensadoun & Weinstein, 1976). The precipitates were centrifuged at 1200 g for 30 min, washed once with 1% (w/v) acetone/triethanolamine and twice with acetone, and then dissolved in the sample buffer (15 mM Tris/HCl, pH 6.8, 0.1% SDS, 1.25% β-mercaptoethanol). Gels were stained with silver (Bio-Rad).

Immunoblotting. After SDS-PAGE, proteins were transferred onto nitrocellulose sheets (Bio-Rad) by the method of Towbin *et al.* (1979). mAbs XVIII G1 and IAI (anti-Hsp60-1 and anti-Hsp60-2; Thole *et al.*, 1988b), mAb 67-2 (anti-Hsp60-2; Anderson *et al.*, 1988), mAb 32TDS15 (anti-antigens 85), mAb

Fig. 1. Purification scheme of the mycobacterial Hsp60. Zinc-deficient culture filtrate in the fixation buffer was applied to a phenyl-Sepharose column and eluted stepwise with buffers of decreasing ionic strength before being applied to an ion-exchange DEAE chromatograph.

2F8-3 (anti-PstS-3; Braibant *et al.*, 1996), mAb F29-47 (anti-19 kDa lipoprotein; Young & Garbe, 1991), mAb CS44 (anti-Hsp60-1) and mAb L7 (anti-Hsp70) were used. mAbs 67-2 and F29-47 were a gift from A. H. J. Kolk (Royal Tropical Institute, The Netherlands). mAbs L7 and CS44 were a gift from L. Walker (UNPD/World/WHO Special Programme for Research and Training in Tropical Disease). Rabbit anti-*M. bovis* BCG serum (Dako) was used at a dilution of 1/200. Alkaline phosphatase conjugated anti-mouse or anti-rabbit immunoglobulins (Promega) were used at a dilution of 1/7500 and 1/5000 respectively.

Purification of Hsp60-2b. Except for hydrophobic chromatography on phenyl-Sepharose, all buffers contained glycerol (0.1%, w/v, final concentration), were adjusted to pH 7.3 and sterilized. All purification steps were performed at 4 °C. The zinc-deficient culture filtrate was dialysed against 20 mM PB and adjusted to 450 mM NaCl. This filtrate was applied to a phenyl-Sepharose column Cl-4B (10 × 30 cm; Pharmacia) (De Bruyn *et al.*, 1987a). The gel was first washed with 20 mM phosphate, 450 mM NaCl buffer (starting buffer) to remove unfixed materials, and then irrigated successively with 20 mM and 4 mM PB, and 10% ethanol. The purification scheme is shown diagrammatically in Fig. 1. Fractions eluted with the 20 mM and 4 mM PB from the phenyl-Sepharose column were further purified by ion-exchange chromatography on DEAE Sephacel and eluted by a 20 to 160 mM phosphate gradient. Fractions showing one band on SDS-PAGE were pooled and concentrated using an Amicon stirred cell equipped with a 10 kDa cut-off membrane (Amicon). Purified recombinant 65 kDa protein (Batches MA-11A and MA-12B) was a gift from J. Van Embden (Bilthoven, The Netherlands) and M. Singh (Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany). *E. coli* GroEL (Batches 13676620 and 14439420) was purchased from Boehringer.

Microsequence analysis of the CNBr peptides obtained from electroblotted proteins. After SDS-PAGE, proteins were electroblotted onto PVDF membranes (Bio-Rad) by the Matsudaira method (Matsudaira, 1987), using a semi-dry blotting apparatus (BioLyon). *In situ* CNBr cleavage of the protein was carried out according to the method of Stone & Williams (1993). Amino acid microsequence analysis was performed by automated Edman degradation using 1–10 pmol peptides on a Beckman LF3400 protein sequencer equipped with an on-line system Gold 126 microgradient HPLC and a

model 168 Diode Array detector (Beckman Instruments). All samples were sequenced using the standard Beckman sequencer procedure 4. The phenylthiohydantoin derivatives were quantitatively identified by reversed phase HPLC on an ODS ultrasphere 5 µm spherical 80 Å pore micro column (2.0 × 250 mm) (Beckman Instruments). All sequencing reagents were from Beckman Instruments.

Extraction of lipids. The different purified Hsp60 proteins (about 100 µg protein ml⁻¹ in 50 mM PB, pH 7.3) were successively extracted four times with chloroform/methanol (2:1, w/v) (Bligh & Dyer, 1959); the two first extractions were performed for 1 h at room temperature, and two other extractions were done for 1 h at 37 °C, with thorough mixing. In each extraction the volume of the organic solvent mixture used was equal to half that of the aqueous phase. The organic phases were pooled and washed once with 0.3% NaCl, twice with distilled water (using a volume of aqueous phase corresponding to 0.2 vol of the organic phase) and then evaporated to dryness under nitrogen. The samples were kept at -30 °C. Lipids from 1 mg purified protein were dissolved in 0.2 ml toluene/acetone (4:1, v/v) for subsequent analyses.

Lipid analysis. Dot blotting was performed on Kieselgel 60W 254 DC Alufolien sheets (Merck). Equal volumes (10 µl) of serial twofold dilutions of the non-covalently bound lipid preparations from the Hsps and standards were used. Lipids were dissolved in toluene/acetone (4:1, v/v) and loaded using a Linomat (Camag) apparatus. Oleic acid (UCB) at an initial concentration of 0.5 µg ml⁻¹ was used as the standard. Plates were sprayed with iodine and the highest dilution giving a positive result was determined in each case. TLC analysis of lipids was performed on the same support as that used for dot blotting. Toluene/acetone (4:1, v/v) was used as developing solvent. TLC plates were stained successively with iodine and α -naphthol/sulfuric acid (5 min at 110 °C). Oleic acid (20 µl of a 200 µg ml⁻¹ solution) was loaded on each chromatographic plate for estimation of R_f variations from one TLC analysis to another. Galactosyl diglyceride from wheat flour (20 µl of a 20 µg ml⁻¹ solution; Sigma) was also loaded on each chromatographic plate as a positive control for staining with α -naphthol/sulfuric acid. Scanning and quantification of the different TLC spots were performed with a GS-670 imaging densitometer (Bio-Rad). Densitometer pictures of the chromatograms were photographed.

To identify free carboxyl-group-containing lipid compounds, samples were treated with a diethylether solution of diazomethane for 1 h at room temperature. The diazomethane was evaporated under nitrogen and the samples were dissolved in toluene/acetone for TLC analysis as described above, and compared with the untreated samples.

Characterization of fatty acids and sugar components by GC. Fatty acid and sugar constituents of lipids were determined by methanolysis. Erythritol (internal standard) was added to lipid extracts derived from about 2 mg Hsp60-2 and the mixtures were treated with 0.75 ml methanolic HCl (1.5 M) for 16 h at 80 °C. The solutions were dried under vacuum over phosphorus pentoxide and potassium hydroxide. For semi-quantitative determination of the relative percentages of fatty acid substituents in the lipid extracts, aliquots of the methanolysates were trimethylsilylated according to Sweeley *et al.* (1963) and analysed by GC, with erythritol as the reference. The remaining methanolysates were partitioned between water and diethylether.

The aqueous phases were dried and the methylglycosides were trimethylsilylated. Fatty methyl esters and sugar derivatives were both analysed by GC and compared to authentic standards, leading to the identification of fatty acid and sugar constituents.

GC. GC was performed on a Girdel G30 apparatus equipped with a fused silica capillary column (25 m length $\times$ 0.32 mm internal diameter) coated with OV-1 (0.3 mm film thickness). A temperature gradient of 100–280 °C (3 °C min⁻¹) was used. The relative percentage of constituents was calculated by comparing the peak height of the various fatty acid methyl esters to that of glucose.

Metabolic labelling with [³H]palmitate. *M. bovis* BCG was grown for 10 d in 25 ml Middlebrook 7H9/ADC medium (Difco) containing 25 μ Ci ml⁻¹ of [9,10(n) ³H]palmitic acid (52 Ci mmol⁻¹; Amersham). Bacteria were harvested by centrifugation and washed with either Tris-buffered saline or PB, depending on the experiment.

Preparation of soluble extracts. Soluble extracts were prepared using a 375 W model Vibra Cell-Sonics Material (Analisis) equipped with a 13 mm solid probe. The cooled bacterial suspension was sonicated for 1 min without interruption, followed by two 5 min pulses (30 s sonication min⁻¹). After centrifugation for 10 min at 12000 g, the supernatant was used for further experimentation.

Analyses of soluble extract. Triton X-114 phase separation was performed according to Bordier (1981). Briefly, Triton X-114 was added to the soluble extract at a final concentration of 2% (v/v). After vigorous mixing, the preparation was first kept on ice and then incubated for 5 min in a 37 °C water bath; the mixture was centrifuged for 5 min at 5000 g and each phase was 'back-washed' (Radolf *et al.*, 1988). The detergent washings of the aqueous phase and the aqueous washings of the detergent phase were added to the detergent phase and the aqueous phase, respectively, before analysis.

For immunoprecipitation mAb 67-2 (anti-Hsp60) or mAb L7 (mc0044; anti-Hsp70) was added to the soluble extract and the mixture was incubated overnight. Then, 100 μ l ProteinA-Sepharose CL-4B (Amersham Pharmacia Biotech) was added to the sample, which was incubated for 1 h at room temperature.

Visualization and identification of radiolabelled components. To visualize radiolabelled components, SDS-PAGE

gels were fixed and treated with 'Amplify' according to the procedures recommended by the manufacturers (Amersham Life Science). Fluorographs were prepared by 3 d exposure to X-ray film (Kodak X-omat-AR) at -70 °C. About 80% of the material was used for autoradiography and 20% for immunoblotting.

Prediction of a tridimensional model of Hsp60-2 and comparison of the hydrophobic character with GroEL protein.

The Hsp60-2 sequence was compared with all sequences in the PDB database (release 79, January 1997) using FASTA (Pearson & Lipman, 1988). Modeller 4.0 program (Sali & Blundell, 1993) was performed on PC586 microcomputers running under Linux. Visualization was performed using WinMGM software (Rahman & Brasseur, 1994) from Ab Initio Technology.

RESULTS

Analysis of the chromatographic fractions eluted from phenyl-Sepharose

The purification scheme applied to the different Hsp60s is shown in Fig. 1. A representative elution profile of zinc-deficient culture filtrates loaded on phenyl-Sepharose is shown on the left of Fig. 2a. The shape and relative size of peaks 5, 6 and 7 were highly reproducible (more than 100 experiments performed over the last 10 years). The unfixed material (peak 4) and the fractions corresponding to the different peaks were analysed by SDS-PAGE. As shown on the left of Fig. 2(b), the major protein constituent of zinc-deficient culture filtrate (lane 3) corresponds to a protein exhibiting an apparent molecular mass of 65 kDa. This protein was identified as Hsp60 by Western blotting (Fig. 2c, left). This protein was a major component of the various chromatographic fractions eluted with buffers of decreasing ionic strength (Fig. 2b, c; left hand side, lanes 5–7). The very faint bands of lower molecular mass present in the SDS-PAGE of fractions 4, 5 and 6 probably correspond to degradation products of the Hsp60 protein as evidenced by the Western blotting experiment (Fig. 2c, left). Western blotting of the different fractions eluted from phenyl-Sepharose with mAb CS-44 (which recognizes Hsp60-1 and not Hsp60-2 used as control; data not shown) gave a pattern similar to that observed with mAb 67-2 which is known to react specifically with Hsp60-2 (Anderson *et al.*, 1988) (Fig. 2c, left), showing that the protein fractions eluted from phenyl-Sepharose contain both Hsp60-1 and Hsp60-2.

SDS-PAGE analysis showed that the unfixed material also contained an Hsp60. To determine whether the presence of Hsp60 in this fraction was due to suboptimal chromatography conditions, i.e. an overloaded phenyl-Sepharose column, or to an intrinsic difference between this protein and the other Hsp60, further chromatography of the unfixed material was performed by loading 0.25 mg protein (ml phenyl-Sepharose gel)⁻¹ (corresponding to about 1/40 of the expected capacity of fixation of this gel); this Hsp60 was again found in the unfixed fraction (Fig. 2b, c; left, lane 4). Consequently, this protein form was named Hsp60-a, and the Hsp60

Fig. 2. Elution pattern on phenyl-Sepharose of the zinc-deficient culture filtrate and soluble cellular extract and electrophoretic analysis of these fractions. In each case, the left of each pair refers to the zinc-deficient culture filtrate and the right is the soluble cellular extract. (a) Phenyl-Sepharose elution pattern; the elution profile was monitored at 280 nm. Peak 4, unfixed fraction; peak 5, fraction eluted with 20 mM PB, pH 7.3; peak 6, fraction eluted with 4 mM PB, pH 7.3; peak 7, fraction eluted with 10% ethanol. (b) SDS-PAGE analysis and Coomassie blue staining of the fractions eluted from the phenyl-Sepharose column. Lanes 1, prestained standards (phosphorylase *b*, 103 kDa; BSA, 77 kDa; ovalbumin, 48 kDa; carbonic anhydrase, 34.2 kDa; soybean trypsin inhibitor, 28.4 kDa), lanes 2, 750 ng Hsp60 and 750 ng 32 kDa protein antigen 85A; lanes 3, 10 μ g protein* from zinc-deficient culture filtrate (left), or soluble cellular extract (right); lanes 4–7, 10 μ g protein* from peak 4 (unfixed fraction) and peaks 5–7 eluted from phenyl-Sepharose. (c) Western blotting analysis of the fractions eluted from the phenyl-Sepharose column after SDS-PAGE and reaction with mAb 67-2. Lanes 1, prestained standards; lanes 2, 0.25 μ g Hsp60 and 0.25 μ g 32 kDa protein antigen 85A; lanes 3, 5 μ g protein* from zinc-deficient culture filtrate (left), or soluble cellular extract (right); lanes 4–7, 5 μ g protein* from peak 4 (unfixed fraction) and peaks 5–7 eluted from phenyl-Sepharose. (d) Western blotting analysis of the fractions eluted from the phenyl-Sepharose column after SDS-PAGE and reaction with mAb 32TDS15. Lanes 1, prestained standards; lanes 2, 0.25 μ g Hsp60 and 0.25 μ g 32 kDa protein antigen 85A; lanes 3, 5 μ g protein* from zinc-deficient culture filtrate (left), or soluble cellular extract (right); lanes 4–7, 5 μ g protein* from peak 4 (unfixed fraction) and peaks 5–7 eluted from phenyl-Sepharose. *This amount of protein was determined before trichloroacetic acid deoxycholate precipitation (see Methods).

forms eluted in the different phenyl-Sepharose fractions were named Hsp60-b, 60-c and 60-d (Fig. 1).

To check that the occurrence of Hsp60s in the different

phenyl-Sepharose fractions was not due to the particular growth conditions, i.e. zinc-deficient culture filtrate, extracts from normal Sauton-grown bacteria harvested during the exponential phase of growth were analysed

Fig. 3. Purity of the Hsp60-2b protein evaluated by SDS-PAGE and silver staining (lanes 1 and 2) and by reaction with mAb 67-2 after transfer from SDS-PAGE to nitrocellulose (lane 3). Lane 1, molecular mass standards; lane 2, 0.25 µg Hsp60-2b; lane 3, 0.15 µg Hsp60-2b.

(Fig. 2, right). The corresponding electrophoretic pattern obtained for the first step of purification was similar to that from the zinc-deficient culture filtrate (Fig. 2a, b, c). Importantly, an entirely different distribution pattern was observed when the P32 protein (antigen 85), the major secreted protein of *M. bovis* BCG, was analysed under the same conditions (Fig. 2d). The P32 protein was present in large amounts in the last fraction only and, in agreement with our previous results (De Bruyn *et al.*, 1987b), the P32 protein was present in much lower amounts in the bacterial extract (2–3%) than in the culture filtrate (15–20%) from which the antigen has been previously purified. Since this antigen family has a higher predicted hydrophobicity (hydrophobicity indexes of antigen 85A, B and C are 0.1382, 0.1437 and 0.1251, respectively) than Hsp60 (hydrophobicity index of Hsp60-2 is 0.0561), it was concluded that the observed distribution of Hsp60 fractions eluted from phenyl-Sepharose was not due to a non-specific adsorption of lipids on these proteins. Taken together, these data indicated that Hsp60 eluted in the different phenyl-Sepharose fractions correspond to various forms of the protein, differing from one another by their hydrophobicity.

The relative amounts of the Hsp60 present in the unfixed fraction and in the three fractions eluted from phenyl-Sepharose loaded with zinc-deficient culture filtrate or normal bacterial extract were estimated by ELISA using purified P64 as the standard and mAb IA1 (anti-Hsp60). This method gave highly reproducible results and showed that the unfixed Hsp60 represented about 5% of the total Hsp60 of the culture filtrate and less than 1% of that of the bacterial extract; each of the two peaks eluted with 20 mM and 4 mM phosphate buffer from the phenyl-Sepharose gel contained roughly 40–45% of the Hsp60 from both sources. The remaining 10% of the Hsp60 was found in the last phenyl-Sepharose fraction. As Hsp60 was present in large amounts in zinc-deficient

culture filtrate and was more easily purified from such culture medium, subsequent work was performed using Hsp60 derived from zinc-deficient culture filtrate.

Purification of Hsp60

Hsp60-2b was also easily purified from peak 5 of the phenyl-Sepharose column (Fig. 2a, left) by ion-exchange chromatography using the previously described procedure for Hsp60-2c (De Bruyn *et al.*, 1987a). As shown in Fig. 3, only one major band was seen on SDS-PAGE after silver staining; the very faint bands of lower molecular mass revealed by Western blotting using mAb 67-2 probably correspond to degradation products often encountered with these Hsp60 proteins. The purification of Hsp60 from peak 7 (Fig. 2a) was less easy than that of Hsp60-2b and c, because of the presence of several major contaminating proteins (see Fig. 2b, lane 7). Nevertheless, after ion-exchange chromatography, Hsp60-2d was sufficiently pure for microsequence analysis after SDS-PAGE and electroblotting.

Microsequence analysis of the CNBr peptides obtained from Hsp60

Direct microsequence analysis of the electroblotted Hsp60-b, -c and -d after *in situ* CNBr cleavage of the Ponceau S stained band led to the unequivocal identification of the N-terminal amino acid sequences of five out of the seven CNBr peptides of the 65 kDa antigen of *M. bovis* (Hsp60-2), starting respectively at positions 2, 165, 192, 287 and 291 as based on the known sequence (Thole *et al.*, 1987) with the following sequences: AKTIAYDEEA, DKVGNEGKIT, RFDKGYISGY, LQD and AILTGGQVIS. The two missing peptides, dipeptides at positions 536 and 539, were probably eluted from the PVDF membrane because of their very small size. No other N-terminal sequences were detected. It follows then that the DEAE chromatography step readily separated Hsp60-2 from Hsp60-1, in agreement with our previous results (De Bruyn *et al.*, 1987a). Consequently, the various Hsp60s purified by ion-exchange chromatography from peaks 5–7 of the phenyl-Sepharose column were renamed Hsp60-2b, -2c and -2d, respectively (Fig. 1).

Overexpression of the Hsp60-2 protein in *E. coli*

To determine whether the occurrence of various Hsp60-2 forms depended on the bacterial source, the protein was overexpressed in *E. coli*. Thus, the soluble extracts of *E. coli* containing pRR3Hsp60-2 or the empty pRR3 plasmid were brought in the starting buffer and applied on a phenyl-Sepharose column at a ratio of 0.5–1 mg protein (ml gel)⁻¹ and submitted to the same stepwise elution as *M. bovis* BCG culture filtrate. In each case, the unfixed fraction and the three phenyl-Sepharose-eluted fractions were analysed by SDS-PAGE, followed by Western blotting using either mAb 67-2, which specifically recognizes the C-terminal part of the myco-

Fig. 4. Electrophoretic analysis of *E. coli* LE392 pRR3Hsp60-2 and *E. coli* LE392 pRR3 extracts on phenyl-Sepharose. (a) Western blotting analysis of the phenyl-Sepharose fractions from *E. coli* pRR3Hsp60-2 extracts and reaction with mAbs 67-2 (anti-mycobacterial Hsp60; a) and XVIIIIG1 (b). (c) SDS-PAGE. In all parts, lanes 1, 0.25 µg GroEL; lanes 2, prestained standards; lanes 3, 0.25 µg Hsp60-2; lanes 4, 40 µg protein from peaks 4–7 of the phenyl-Sepharose column (see the legend of Fig. 2a). The amount of protein was determined before trichloroacetic acid deoxycholate precipitation (see Methods).

bacterial Hsp60-2, or mAb XVIIIIG1, which recognizes both the *E. coli* GroEL and the mycobacterial Hsp60-2 (Fig. 4). As expected, a protein band corresponding to the mycobacterial Hsp60-2 was clearly seen in each lane, except in lane 1 since the *E. coli* GroEL doesn't react with the mAb 67-2 (Fig. 4a). When the same experiment was performed using the control *E. coli* strain containing empty pRR3 (Fig. 4b, c), only *M. bovis* BCG Hsp60-2 control gave a band (Fig. 4b, lane 3). Interestingly,

Western blotting using mAb XVIIIIG1, which recognizes both Hsp60 and GroEL, showed that the *E. coli* GroEL exhibits the same hydrophobic behaviour as *M. bovis* BCG Hsp60-2 (Fig. 4c). It has to be noted, however, that the overexpression of Hsp60 in *E. coli* led to much degradation of this protein (Fig. 4a, lanes 4–6), especially in Hsp60-2 of the unfixed fractions (Fig. 4a, c). As a control for the results presented in Fig. 4c, the behaviour of commercially available GroEL on phenyl-Sepharose was analysed. Passage of GroEL in the starting buffer through a phenyl-Sepharose column resulted in extensive degradation (90 %) of the protein (data not shown). The remaining 10 % of the intact GroEL was recovered in the two most hydrophobic fractions eluted from the phenyl-Sepharose column, i.e. peaks 6 and 7; trace amounts of GroEL were found in peak 5 and it was just detectable in the unfixed phenyl-Sepharose fraction (peak 4). The observed instability of GroEL was not due to storage conditions, since incubation of the protein at 37 °C for 3 h at a concentration of 1 mg ml⁻¹ did not affect the protein concentration and, more importantly, only one band of the same molecular mass as the non-incubated control was detected by SDS-PAGE analysis. It was thus concluded that the passage of GroEL through the phenyl-Sepharose column was responsible for the observed degradation of purified GroEL.

Evidence for the association of non-covalently bound lipids to purified Hsp60 proteins

The existence of various Hsp60-2s differing by their hydrophobicity may be due to several factors, of which the association of different lipids to these proteins is one. This possibility was explored by extracting the various purified Hsp60s with organic solvents and estimating the amounts of extracted lipids from each Hsp60 form by dot blotting. Expressed as µg equivalents of oleic acid, about 3.5 and 7 µg lipids were extracted from 100 µg Hsp60-2b and Hsp 60-2c, respectively, and about 3.5 µg and 1 µg lipids were extracted from 100 µg recombinant Hsp60-2 and GroEL, respectively. These data clearly showed that significant amounts of lipids were non-covalently associated to all the purified proteins, and help us in determining the volume of lipid sample to be used in TLC analyses (see below).

Analysis of the non-covalently bound lipids associated with the purified Hsp60 proteins

Lipids extracted from the two major Hsp60-2s, recombinant Hsp60-2 and GroEL were analysed by TLC using toluene/acetone (80:20, v/v) as the developing solvent. The TLC profiles of the different samples were very similar (Fig. 5); at least ten lipid spots were visualized with iodine. Among these, four spots were stained with the α -naphthol/sulfuric acid reagent, suggesting that they corresponded to glycolipids. The migration of one of these spots (R_F 0.43) was shifted following the methylation of the lipid extracts (R_F around 0.50), suggesting the occurrence of some esterifiable groups in

Fig. 5. TLC of the non-covalently bound lipids associated with the various Hsps. Lipids were extracted from Hsp60-2a, -2b, the recombinant Hsp60-2 expressed in *E. coli* (Rec) and *E. coli* GroEL. The amounts of lipids analysed by TLC were estimated on the basis of the amounts of lipids extracted from the various Hsps. Oleic acid was used as standard and the plate was stained with iodine. The asterisks indicate glycolipids

Table 1. Relative percentages of fatty acid methyl esters obtained by methanolysis of lipids extracted from purified Hsp60 proteins and analysed by GC

Percentages shown are the mean values of three independent experiments. The error was estimated to be $\pm 10\%$.

Fatty acid	Hsp60-2b	Hsp60-2c (65 kDa)
C _{14:0}	—	6.8
C _{16:0}	33	43
C _{18:0}	56	22
C _{18:1}	11	19
C _{19:0}	—	8

this compound. Furthermore, and most importantly, no spot was stained with the Dittmer–Lester reagent (Dittmer & Lester, 1964), a specific spray for the detection of phospholipids. The nature of the ester-linked fatty acid residues was determined by GC analysis of the methanolysis products of the lipids extracted from the different Hsp proteins. Fatty acids C_{16:0}, C_{18:0} and C_{18:1} were the main fatty acyl substituents detected in all the methanolysates, C_{16:0} being the most abundant component (Table 1). In addition, tuberculostearate (10-methyl octadecanoate) was present in the methanolysis products of lipids from Hsp60-2c. Glucose was the only abundant sugar constituent identified by GC in the methanolysis products of the four lipid samples. Interestingly, glucose represented (in relative percentage) 19 ± 1 and $41 \pm 2\%$ of the methanolysis product constituents from Hsp60-2c and Hsp60-2b, indicating that the various Hsp60-2s may also differ in their glycolipid content. Results presented in Table 1 are limited to the

comparison of mycobacterial Hsp60-2b and Hsp60-2c fatty acid methyl esters contents as the recombinant Hsp60-2 and GroEL proteins were produced in a different host and their purification did not include a phenyl-Sepharose step.

Labelling of Hsp60 with palmitate

To confirm the association of lipids with Hsp60, mycobacteria were labelled with palmitate, and the resulting protein extracts subjected to phase separation in Triton X-114 (Bordier, 1981) and analysed. The positive controls for phase separation consisted of two well-known mycobacterial lipoproteins, i.e. the 19 kDa and 40 kDa lipoproteins. As expected, these two lipidated proteins were found exclusively in the detergent phase (Fig. 6b, lanes 6 and 7) and the majority of the soluble-extract proteins were present in the aqueous phase (Fig. 6a, lane 6). Although small amounts of labelled Hsp60 were found in the detergent phase, most of the Hsp60 protein was observed in the aqueous phase where it appeared to be the unique labelled protein (Fig. 6a, lane 2). Immunoprecipitation experiments using the anti-Hsp60 mAb 67-2 and the anti-Hsp70 mAb L7 and Protein A/Sepharose further substantiated the above observation. As shown in Fig. 6(c, d), a labelled band was seen on the SDS-PAGE gel and identified by Western blotting as Hsp60-2 (Fig. 6c, lane 4) whereas no Hsp70 was detectable in the fraction (Fig. 6d). These clearly demonstrated the specific association of lipids with Hsp60. The predilection of this protein for the aqueous phase was not surprising in view of the tridimensional model of Hsp (see below) and may be explained by the hydrophilic character of the external surface of this oligomeric protein; the specific association of radiolabelled palmitate with the protein is also in agreement with the presence of a central hydrophobic channel in Hsps where lipids could be buried, as illustrated below. The presence of a tiny amount of Hsp60 in the detergent phase may be due to an extraction of a small portion of this major extract protein in the hydrophobic phase.

Prediction of a tridimensional model for Hsp60-2

To better understand the origin of the dual behaviour of Hsp60 in the Triton X-114 experiment, a three-dimensional model of the protein was built using Modeller, software that calculates a structure from a primary sequence using the bacterial GroEL (PDB code: 1der) as a template. The GroEL protein has approximately 59% sequence identity and 84% sequence homology with 539 residues of the Hsp60-2 sequence and is made of fourteen identical subunits (Braig *et al.*, 1994). Most of the residues involved in ATP binding are conserved, particularly for segment Asp86–Thr90. Only the C-terminal part, from Glu522 to Phe539 of the Hsp60-2 sequence, was not built due to the lack of data in the same portion of GroEL in its pdb datafile.

Molecular hydrophilic potentials (Brasseur, 1991) were

Fig. 6. Analysis of radiolabelled extracts from phase separation in Triton X-114 and affinity immunoprecipitation. (a) Analysis by SDS-PAGE, autoradiography and immunoblotting of the Triton X-114 aqueous phase. Lanes 1 and 3, prestained standards; lane 2, autoradiography of the aqueous phase; lane 4, 0.15 μ g Hsp60; lanes 5 and 6, Western blotting of the aqueous phase with mAbs IA1 (anti-Hsp60) (lanes 4 and 5) and polyclonal rabbit anti-*M. bovis* (lane 6). (b) Analysis of the Triton X-114 detergent phase. Lanes 1, 3, 4, as (a); lane 2, detergent phase autoradiography; lanes 5–7, Western blotting of detergent phase with mAbs IA1 (anti-Hsp60) (lanes 4 and 5), F29-47 (anti-19 kDa mycobacterial lipoprotein) (lane 6) and 2F8-3 (anti-40 kDa mycobacterial PstS-3) (lane 7). (c) Analysis by SDS-PAGE, autoradiography and immunoblotting of immunoprecipitated Hsp60. Lanes 1–3 as (a); lane 2, autoradiography of immunoprecipitated Hsp60; lane 4, Western blotting with mAb 67-2. (d) Analysis by SDS-PAGE, autoradiography and immunoblotting of immunoprecipitated Hsp70. Lanes 1 and 3, as (a); lane 2, autoradiography of immunoprecipitated Hsp70; lane 4, Western blotting with mAb L7.

Fig. 7. Comparison of the molecular hydrophobic potentials of five subunits of the 'double ring cylinder' in GroEL and Hsp60-2. The top row shows macromolecules from the inside of the cavity, bottom views from the outside. Orange surfaces are associated with hydrophobic isopotentials and green patches with hydrophilic isopotential domains. For the cavity, molecular hydrophobic potentials of subunit A are displayed in blue (hydrophilic patches) and red (hydrophobic patches).

displayed for Hsp60-2 and GroEL structures (Fig. 7). This calculation allows visualization of the distribution of hydrophobic and hydrophilic domains of the molecules. The hydrophobicities of GroEL and Hsp60-2

assemblies are very slightly different. Considering the sequence conservation and hydrophobicity between GroEL and Hsp60-2, we can suggest that their macromolecular features are globally identical. However, their

inside cavities are more hydrophobic than their respective outer parts. This feature of the GroEL binding surface cavity was previously reported by Frydman & Hartl (1996).

DISCUSSION

M. bovis BCG possesses, like *Mycobacterium tuberculosis*, two *hsp60* genes. The first of the two genes to be sequenced was *hsp60-2* (Thole *et al.*, 1987) as in *M. tuberculosis* (Shinnick, 1987). The two Hsp60-2 proteins are identical. The product of the *hsp60-2* gene, expressed as a recombinant protein, has been extensively studied for its immunological properties (Thole *et al.*, 1988b). The gene encoding Hsp60-1 in *M. tuberculosis* has been identified and was sequenced later. The coding regions of the *hsp60-1* gene of *M. bovis* BCG have identical restriction maps to those of the *M. tuberculosis hsp60-1* gene and produce similar amplicons when amplified using PCR and eight primers (Kong *et al.*, 1993). Hsp60-1 displays 61% amino acid sequence identity with Hsp60-2; this latter is closer to *E. coli* GroEL than Hsp60-1. We have previously shown that large amounts of Hsp60 are released into the culture filtrates of *M. bovis* BCG grown on zinc-deficient Sauton medium (De Bruyn *et al.*, 1987a). Chromatography of the macromolecules from these filtrates on hydrophobic phenyl-Sepharose, using a stepwise elution with buffers of decreasing ionic strength, now reveals the occurrence of Hsp60 in the different fractions (renamed Hsp60-a, -b, -c and -d). Western blotting demonstrated the presence of both Hsp60-1 and -2 in these fractions. Further purification of Hsp60 by ion-exchange chromatography led to the isolation of the various Hsp60-2s as evidenced by N-terminal amino acid microsequence analyses of the CNBr peptides from the two major forms, Hsp60-2b and -2d. The same protein forms were observed when mycobacterial Hsp60-2 was overexpressed in *E. coli*. The differing abilities of these proteins to adsorb to phenyl-Sepharose gels at relatively low ionic strength, to elute from the gels using buffers of decreasing hydrophilicity, and, more importantly, their high affinity for phosphatidylcholine liposomes (J. M. Ruyschaert, personal communication) suggest that they differ one from another in terms of hydrophobicity. Since no lipoprotein consensus motif is present on the Hsp60 protein sequence, we hypothesized that different amounts and/or kinds of lipids could be closely associated with these identical polypeptide chains. Consequently, the two major purified proteins Hsp60-2b and -2c were extracted with organic solvents and the resulting non-covalently associated lipids were compared to those associated with recombinant 65 kDa protein of *M. bovis* BCG expressed in *E. coli* and to the *E. coli* GroEL. In agreement with our hypothesis, lipids were found associated with all the purified proteins examined and exhibited a qualitatively similar profile on TLC. Furthermore, different amounts of non-covalently associated lipids were extracted from the various proteins. As expected from the above assumption, Hsp60-2c, which was eluted at a lower ionic strength

than Hsp60-2b, contained more lipids than Hsp60-2b. That the different fractions did not represent loss of lipids from one single complex was demonstrated by the lipid composition of the various purified Hsp60-2s. While C_{16:0} was the major fatty acid constituent of Hsp60-2c, the homologous Hsp60-2b contained less of this fatty acid than C_{18:0}; conversely, this latter protein contained twofold more glucose-containing lipids than the former Hsp60-2 form.

Indeed, the occurrence of non-covalently bound lipids associated with Hsp60 was not due to an artefact since different sources and different purification schemes have been applied to the isolation of the natural BCG Hsp60-2, the BCG 65 kDa recombinant protein expressed in *E. coli*, and *E. coli* GroEL. The existence of the phenyl-Sepharose Hsp60 forms was dependent neither on the presence of zinc in the growth medium, nor on the bacterial compartment from which the proteins were isolated (culture filtrate or cell extract), nor on the bacterial source (*M. bovis* or *E. coli*). In addition, not all the proteins exhibit this behaviour on phenyl-Sepharose; for instance, the major secreted protein of *M. bovis*, P32 (antigen 85), which has a predictable hydrophobicity that is higher than that of Hsp60, was eluted from the phenyl-Sepharose column in a major fraction, proving that the hydrophobic behaviour of Hsp60 was not due to a non-specific adsorption of lipids on proteins. This conclusion was further supported by the absence of phospholipids in the lipids extracted from the purified Hsp proteins; these substances, which include the mannose-containing phosphatidyl inositol, are known to be present not only in the plasma membrane but also in the external envelope of mycobacteria (Ortalo-Magné *et al.*, 1996) and thus represent sensitive controls for the absence of non-specific adsorption of lipids on proteins. The detection of glucose, but not mannose, as the unique sugar constituent of the methanolysis products of lipids extracted from Hsp60 was in agreement with the absence of phospholipids in Hsp lipids. Furthermore, no typical mycobacterial lipid could be detected in lipid extracts from Hsp60, another strong argument pointing towards the absence of non-specific adsorption of lipids. The very similar nature of the lipids extracted from the Hsp60 from the phylogenetically unrelated Gram-negative *E. coli* and the Gram-positive *M. bovis* suggests a common biological function of these lipids. The evidence of a selective labelling of Hsp60 with palmitate, but not Hsp70, reinforced the concept of a specific association of lipids to these Hsp60 proteins. Besides, the study brings an answer to an unexplainable result previously reported by Young & Garbe (1991). To identify lipoproteins of *M. tuberculosis* H37Rv, the authors performed two complementary experiments. On the one hand, they analysed the distribution of proteins from a Triton X-114-treated soluble extract of *M. tuberculosis* between the aqueous and detergent phases. In this experiment, the 65 kDa protein was found in the aqueous phase as expected for this water-soluble protein. On the other hand, they cultivated *M. tuberculosis* in the presence of [³H]palmitate and delipi-

dated the labelled bacteria with chloroform/methanol prior to disruption of the bacterial cells. Proteins of the extract prepared from the delipidated bacteria were analysed by two-dimensional SDS-PAGE and fluorography. Following these analyses, the authors identified some well known lipoproteins and reported a strongly labelled signal which overlapped with the 65 kDa antigen spot; they could not explain the origin of this [³H]palmitate signal and have proposed that 'occurrence of a lipoprotein with similar electrophoretic mobility to the 65-kDa antigen remained an attractive possibility'. Our data showed that the radioactive spot described in the experiment of Young & Garbe corresponds to the 65 kDa Hsp60 protein.

The existence of an hydrophobic channel, as visualized in the model, and consisting of hydrophobic amino acids (conserved in mycobacterial Hsp60) at the entrance of the cavity provides a suitable interface for interaction with lipids. The production of stable, purified lipid-associated Hsp60-2 protein complex is difficult. Moreover the stability of these complexes might be different in mycobacteria and *E. coli*, and vary with the nature of the Hsp60 (1 or 2). These facts could possibly be related to the very low yield of associated lipids found in *E. coli* GroEL. For these reasons, the role of these non-covalently bound lipids in protein folding can presently only be speculative and restricted to the *in vivo* situation. Distribution of the different Hsp60 proteins and the better stability of the most hydrophobic fractions has been reproducibly observed for years. This suggests to us that the less-lipidated Hsps with a higher glycolipid content (as based on the higher glucose content of lipids extracted from Hsp60-2b compared to Hsp60-2c) would bind more hydrophilic proteins and contribute to facilitating their folding; conversely, the more lipidated Hsp60-2 would participate in the folding of the more hydrophobic proteins. Possibly the involvement of lipids and glycolipids in the *in vivo* protein folding could somehow improve the yield of biologically active molecules produced.

The similar hydrophobic behaviour shown for mycobacterial Hsp60-2 and Hsp60-1 by using specific antibodies, and for *E. coli* GroEL in the overexpression control experiment may indicate that the presence of non-covalently associated lipids could be a general feature of cylindrical chaperones. An important input of work 20 years ago demonstrated the requirement of lipid for cytochrome oxidase activity (Vik & Capaldi, 1977), followed by the demonstration of the necessity of the presence of phospholipids for the activity of numerous membrane enzymes; why would lipids not play a role in such a complex function as protein folding?

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