Autophosphorylation of the 16 kDa and 70 kDa antigens (Hsp 16·3 and Hsp 70) of Mycobacterium tuberculosis

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Several antigens of Mycobacterium tuberculosis, identified by monoclonal antibodies, have been previously cloned and are being exploited in the development of improved vaccines and diagnostic reagents. In this study, the molecular characteristics of two of these antigens, the immunodominant proteins Hsp 16·3 and Hsp 70, were analysed in further detail by assessing their capacity to undergo protein phosphorylation, a chemical modification frequently used by organisms to adjust to environmental variations. Hsp 16·3 was overproduced in an Escherichia coli expression system and purified to homogeneity. Upon incubation in the presence of radioactive ATP, it was shown to possess autophosphorylation activity. Two-dimensional analysis of its phosphoamino acid content revealed that it was modified exclusively at serine residues. In addition, cross-linking experiments demonstrated that it could tightly bind to ATP. Purified Hsp 70 was also shown to autophosphorylate but phosphorylation occurred exclusively at threonine residues. This reaction was found to be strongly stimulated by calcium ions. These data indicate that both structural and functional similarities exist between Hsp 16·3 (Acr) and α-crystallin, a eukaryotic protein which plays an important role in maintaining the transparency of the vertebrate eye, and that the functional properties of Hsp 70 from M. tuberculosis are similar to those of other bacterial members of the Hsp 70 family, particularly the E. coli homologue DnaK.

INTRODUCTION

Mycobacterial diseases remain a major cause of human morbidity and mortality. Epidemiological data indicate that up to one-third of the world’s population is latently infected with Mycobacterium tuberculosis, and about 1·5 to 2 million people die from tuberculosis annually (Olsen & Andersen, 2003; Sudre et al., 1992). The virulence of this intracellular pathogen lies, in particular, in its capacity to persist within the host in a stationary-phase-like dormant state for extended periods before reactivating to cause clinical disease (Wayne, 1994). During latency, M. tuberculosis is thought to reside in inflamed and necrotic tissue in pulmonary granulomas (Dannenberg, 1993). The molecular mechanisms used by M. tuberculosis to remain latent within the host and to infect macrophages and establish disease are still poorly understood. In recent years, effort has been directed towards the characterization of the mycobacterial antigens involved in interactions within the host immune system (Olsen & Andersen, 2003; Verbon et al., 1992). A series of prominent antigens from M. tuberculosis have been identified using murine monoclonal antibodies, including two proteins with molecular masses of 70 kDa and 16 kDa.

The 16 kDa protein has striking sequence similarity to the small heat-shock protein Hsp 16·3 (Acr) (Chang et al., 1996). This immunodominant antigen also has sequence similarity to α-crystallin, a protein that plays an important role in maintaining the transparency of the vertebrate eye (Groenen et al., 1994; Horwitz, 1992). In addition, it has been reported to act as an ATP-independent chaperone with a complex oligomeric active structure consisting of a trimer of trimers (Chang et al., 1996; Yuan et al., 1996). Interestingly, this antigen is undetectable during exponential growth of M. tuberculosis, but is overproduced during the stationary phase (Yuan et al., 1996), as well as in adverse conditions such as oxygen deprivation, nutrient depletion, low pH or accumulation of toxic by-products (O’Toole et al., 2003). The 70 kDa protein resembles the heat-shock protein Hsp 70, homologues of which have attracted attention because they are immunodominant antigens for both T- and B-cell responses in humans and infected animals (Roche et al., 1994). However, their function remains unclear, as does the significance of the immunological cross-reactivity between human and mycobacterial Hsp 70 molecules. Besides inhibition of antigen processing (Moreno et al.,...
1988), *M. tuberculosis* employs various strategies for survival in host cells, including inhibition of phagosome–lysosome fusion, lysosomal acidification and inhibition of interferon-γ activation (Koul et al., 2001). These different mechanisms of protection rely on the ability of the pathogen to adapt to changes in its environment and, therefore, to trigger regulatory processes that are critical in pathogenesis.

Protein phosphorylation catalysed by protein kinases is a frequent post-translational modification used by organisms to transduce extracellular signals into cellular functions. Its occurrence was first demonstrated in eukaryotes, then later in prokaryotes, and it is now considered a universal modification of proteins allowing all living systems to respond to environmental variations (Hunter, 1995). In the case of *M. tuberculosis*, the presence of proteins possessing phosphorylated tyrosine residues has been detected using an antiphosphotyrosine antibody, but none of them has been identified (Chow et al., 1994). The analysis of the total genome sequence of the bacterium has suggested the presence of eleven putative protein kinases and four phosphoprotein phosphatases (Cole et al., 1998). Recently, two protein tyrosine phosphatases (Koul et al., 2000) and five serine/threonine kinases, PknD (Peirs et al., 1999), PknB (Av-Gay et al., 1999), PknF, PknG (Koul et al., 2001) and PknA (Chaba et al., 2002), have been cloned and characterized. However, the biological function of these enzymes has not been determined, with the exception of PknA, which might have a regulatory role in cell division (Chaba et al., 2002).

As Hsp 16.3 and Hsp 70 are major antigens in mycobacterial infections and because of the presence of protein phosphorylating activities in *M. tuberculosis*, we examined the possibility that these proteins could undergo phosphorylation, and thus whether such modifications should be taken into account when deciphering the molecular pathogenesis of tuberculosis.

**METHODS**

**Bacterial strains and plasmids.** *Mycobacterium tuberculosis* H37Rv (ATCC 27294) was used as template for PCR amplification of the *hsp16.3* gene. *Escherichia coli* DH5α was used to propagate plasmids in cloning experiments. *E. coli BL21(DE3) was used for expression experiments. The plasmid vector pET15 was purchased from Novagen.

**Culture media and growth conditions.** *E. coli* strains were grown in LB or 2YT medium at 37°C. For strains carrying drug-resistance genes, ampicillin was added to the medium at 50 μg ml⁻¹. Cultures of *M. tuberculosis* were grown in Dubos medium at 37°C. Growth was monitored by measuring OD₆₀₀. The cells were collected in stationary phase.

**Preparation of total cellular extracts.** For preparing cellular extracts of *M. tuberculosis*, bacteria were first collected by centrifugation. The pellet was washed in 20 mM MOPS at pH 7.5, 5 mM NaCl, 1 mM EDTA and 1 mM DTT, and cells were resuspended in the same buffer, then subjected to mechanical bead beatage for 2 × 25 s. To remove cell debris, extracts were centrifuged at 14 000 g for 30 min. The resulting supernatant was filtered and glycerol was added to a final concentration of 10% (v/v). Cell lysates were split into aliquots and frozen at -70°C.

**Two-dimensional separation of proteins.** Proteins (about 200 μg) from a whole-cell extract of *M. tuberculosis* were analysed using the O’Farrell gel technique (O’Farrell et al., 1977). Separation in the first dimension was achieved by non-equilibrium isoelectric focussing in 4% acrylamide and ampholines in the pH range of 3–10 in the presence of 9.5 M urea. Separation in the second dimension was performed by SDS-PAGE in 12.5% polyacrylamide. After protein separation, gels were soaked in 16% trichloroacetic acid and heated for 10 min at 95°C. Proteins were then visualized by silver staining, and phosphoproteins were detected by autoradiography. Radioactive spots were excised from the gel and analysed by mass spectrometry.

**Identification of proteins by mass spectrometry.** Protein spots of interest were cut from the gel using a pipette tip, placed in 0.5 ml microcentrifuge tubes, and processed as described by Couchesnes & Patterson (1999) with some modifications. The gel was washed once with MilliQ water and twice with 25 mM NH₄HCO₃, then destained and dehydrated by washing three times (~5 min each) with a solution containing equal volumes of 50 mM NH₄HCO₃ and acetonitrile. The destained gel pieces were rehydrated twice with 50 mM NH₄HCO₃ and proteins were reduced by incubation with 20 mM DTT in 50 mM NH₄HCO₃ for 1 h at 37°C, then washed again briefly with 50 mM NH₄HCO₃. The proteins were alkylated with 25 mM iodoacetamide in 50 mM NH₄HCO₃ and incubated at room temperature in the dark for 30 min, washed twice with 50 mM NH₄HCO₃ and dehydrated three times with acetonitrile (~5 min each). The gel was left to dry at room temperature and stored at -70°C. The gel was digested overnight at 32°C with a minimal amount (~10 μl) of ice-cold 5 mM NH₄HCO₃ containing 2 μg trypsin μl⁻¹. The peptide samples were analysed by addition of 0.1 vol. 2% trifluoroacetic acid prior to mass spectrometry. Thin layer matrix surfaces of x-cyano-4-hydroxycinnamic acid mixed with nitrocellulose were prepared as described by Shevchenko et al. (1996). The acidified digest was loaded onto the thin layer and allowed to dry prior to rinsing with water. A Reflex III matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker Daltonik) equipped with a Scout-384 probe was used to obtain positive ion mass spectra of digested proteins. Peptide mass fingerprints were searched against the non-redundant database (available from the National Center for Biotechnology Information). Partial enzymic cleavage sites, oxidation of methionine, pyrogglutamic acid formation at N-terminal glutamine and modification of cysteine by acrylamide were considered in these searches.

**Isolation of chromosomal DNA from *M. tuberculosis*.** One colony of *M. tuberculosis* was inoculated into 10 ml Dubos medium containing the appropriate supplements and incubated at 37°C. After growth for 1–2 weeks, bacteria were subcultured into 150 ml Dubos medium. The culture was grown to an OD₆₆₀ of 0.7–1.0, and 0.1 vol. 2 M glycerol was added 20 h before harvest. Bacteria were pelleted by centrifugation at 16 000 g for 20 min at 4°C and the cell pellets were resuspended in 2 ml TE (10 mM Tris/HCl, pH 7.5, 1 mM EDTA) then divided into 500 μl aliquots. The tubes containing the cell suspensions were incubated at 80°C for 1 h to kill the bacteria. A mixture containing 50 μl lysosome and lipase (2 mg ml⁻¹ each in TE) and 5 μl D Nase-free RNase (0.5 μg ml⁻¹) was added to each suspension and incubated at 37°C for 2 h. Tubes were then frozen in an ethanol/dry ice bath for 10 min followed by incubation at 75°C for 10 min. After cooling to room temperature, proteinase K and SDS were added to final concentrations of 500 μg ml⁻¹ and 0.5% (w/v), respectively, and the mixture incubated at 50°C for 1 h. The solution was extracted twice with 0.5 ml...
phenol/chloroform/isooamyl alcohol (25/24/1, by vol.) and once with 0-5 ml chloroform. Chromosomal DNA present in the aqueous phase was precipitated by the addition of 0-02 vol. 5 M NaCl and 2 vols 99% ethanol at room temperature. The chromosomal DNA was collected by centrifugation at 11 600 g for 10 min at 4 °C, then washed twice with 1 ml 70% ethanol. After removing traces of ethanol by brief centrifugation, the DNA pellet was air-dried at room temperature, dissolved in 300 µl TE and stored at 4 °C. The amount of DNA was determined by measuring A260 in a Thermo Unicam UV2 spectrophotometer, and chromosomal DNA was analysed by agarose gel electrophoresis.

**Expression and purification of Hsp 16 3.** The hsp16-3 gene was amplified, with appropriate restriction endonuclease cleavage sites at both ends, from genomic DNA from *M. tuberculosis* H37Rv using the primers 5'-GGA ATT CCA TAT GCC CAC CAC CCT TCC CGT TC-3' and 5'-CCG GGA TTC TCA GGT GGT GGA CCG GAT CTG-3'. The amplified fragment was subcloned into the pET15 expression vector to yield the plasmid pET-hsp16-3. The nucleotide sequence of the amplified gene was checked by dideoxynucleotide sequencing (Sanger et al., 1977). The pET-hsp16-3 expression plasmid was used to transform competent *E. coli* BL21(DE3) cells. Overproduction of the 6His-Hsp16-3 protein was obtained by induction with 1 mM IPTG. After 2 h, 6His-Hsp 16-3 was extracted and purified using an immobilized Zn2+ matrix, suitable for purification of fusion proteins carrying a polyhistidine tag. Production of the 6His-Hsp 16-3 protein was confirmed by analysis of Coomassie blue-stained polyacrylamide gels. Protein concentration was determined using the Coomasie Plus Protein Assay (Pierce).

**In vitro phosphorylation assay.** In vitro phosphorylation of about 5 µg purified 6His-Hsp 16-3 protein or Hsp 70 protein (Sigma) was performed for 10 min at 37 °C in 20 µl of a buffer containing 25 mM Tris/HCl (pH 7.0), 1 mM DTT, 1 mM EDTA, 5 mM MgCl2 and 200 µCi (7.4 MBq) [γ-32P]ATP ml⁻¹. In some assays, MgCl2 was replaced with 5 mM MnCl2, ZnCl2 or CaCl2. In each case, the reaction was stopped by addition of an equal volume of 2 × sample buffer (Laemmli, 1970). After electrophoresis, gels were soaked in 16% trichloroacetic acid for 10 min at 90 °C. They were stained with Coomassie blue and radioactive proteins were visualized by autoradiography.

**Analysis of the phosphoamino acid content of proteins.** Protein samples were separated by one-dimensional gel electrophoresis (Laemmli, 1970), then electroblotted onto an Immobilon PVDF membrane. Phosphorylated proteins bound to the membrane were detected by autoradiography. The 32P-labelled protein bands were excised from the Immobilon blot and hydrolysed in 6 M HCl for 1 h at 110 °C. The acid-stable phosphoamino acids thus liberated were separated by electrophoresis in thefirst dimension at pH 1–9 (800 V h) in 7.8% acetic acid/2.5% formic acid, followed by ascending chromatography in the second dimension in 2-methyl-1-propanol/formic acid/water (8/3/4, by vol.). After migration, radioactive molecules were detected by autoradiography. Authentic phosphoserine, phosphothreonine and phosphotyrosine were run in parallel and visualized by staining with ninhydrin (Duclos et al., 1991).

**ATP binding.** Photofluorimetry [γ-32P]ATP cross-linking experiments were performed as described by Matsuyama et al. (1990). The 6His-Hsp 16-3 protein was incubated in a buffer containing 25 mM Tris/HCl, pH 7–0, 1 mM DTT, 1 mM EDTA, 5 mM MgCl2 and [γ-32P]ATP (3000 Ci mmol⁻¹; 110 TBq mmol⁻¹) at 37 °C for 10 min. The reaction was stopped by placing the mixture on ice. The mixture was then subjected to affinity cross-linking for 45 min at 4 °C using a 254 nm lamp at a distance of 4 cm. Cross-linking reactions were carried out with or without UV irradiation in the presence or absence of ATP at various concentrations. The reaction was stopped by addition of 2 × Laemmli buffer. After boiling, samples were subjected to SDS-PAGE in 10% polyacrylamide gels and radioactive bands were visualized by autoradiography.

**RESULTS**

**Electrophoretic analysis of total phosphoproteins.** Total protein extracts were prepared from *M. tuberculosis* H37Rv and incubated with [γ-32P]ATP. Phosphorylated proteins were detected by autoradiography after two-dimensional separation and silver staining. The results presented in Fig. 1 show that five major phosphoproteins (indicated by arrows) were detected by both staining and autoradiography, including three proteins with molecular masses of 70, 60 and 20 kDa, and two proteins with a similar molecular mass of about 16 kDa. To characterize these proteins, the corresponding spots were excised from the gel and analysed individually by mass spectrometry. The results indicated identity of the 70 kDa phosphoprotein (spot 1) with Hsp 70 and of the two phosphoproteins of about 16 kDa (spot 4 and 5) with Hsp 16-3. The identity of spots 2 and 3 could not be determined. To further analyse the phosphorylation of Hsp 16-3 and Hsp 70 it was necessary to overproduce and purify the Hsp 16-3 protein; Hsp 70 could be obtained commercially.

**Overproduction and purification of Hsp 16-3**

The hsp16-3 gene lacking the start codon was synthesized by PCR and cloned in plasmid pET15. The resulting plasmid pET-hsp16-3 encoded a fusion protein consisting of the entire Hsp 16-3 protein with 11 extra amino acids, including six histidine residues, at its N-terminus. This construct was used to transform competent *E. coli* BL21(DE3). Upon induction with 1 mM IPTG, efficient overproduction of a 16 kDa protein, consistent with the calculated molecular mass of the fusion protein 6His-Hsp 16-3, was obtained in the soluble fraction from the cells (Fig. 2, lane 2). As expected, no overproduction of this protein was observed in control cells grown in the absence of IPTG (Fig. 2, lane 1). The 6His-Hsp 16-3 fusion protein was then purified to homogeneity in a single-step affinity chromatography procedure using an immobilized Zn2+ matrix (Fig. 2, lane 3). About 1 mg pure protein could be obtained from 100 ml bacterial culture.

**Autophosphorylation of Hsp 16-3 at serine**

The 6His-Hsp 16-3 protein was purified by chromatography and assayed for phosphorylation. Upon in vitro incubation in the presence of [γ-32P]ATP, this protein was intensely labelled (Fig. 3, lane 2), indicating that it contained an intrinsic protein kinase activity able to catalyse its own phosphorylation. The phosphoamino acid content of the labelled protein was then determined after hydrolysis and two-dimensional analysis. In the conditions used, only acid-resistant phosphoamino acids could be analysed, a number...
of other phosphorylated compounds, such as phosphohistidine, phosphoarginine and phosphoaspartate, being labile in acid. Only phosphoserine could be detected on the corresponding autoradiogram (Fig. 4a), which showed that 6His-Hsp 16·3 was modified exclusively at serine residues.

### Binding of ATP to 6His-Hsp 16·3

To test the ability of ATP to bind to Hsp 16·3, a UV cross-linking assay was performed. The 6His-Hsp 16·3 protein was incubated with [γ-32P]ATP and the formation of cross-linked-ATP following irradiation at 254 nm was analysed by SDS-PAGE and autoradiography. The results showed that ATP could bind effectively to 6His-Hsp16·3 to yield a stable radioactive complex (Fig. 5, lane 3). Under the same conditions of incubation and irradiation, a control
experiment showed, by contrast, that no cross-linking occurred between bovine serum albumin and ATP (Fig. 5, lane 2).

**Autophosphorylation of Hsp 70 at threonine**

For comparison with the Hsp 70 homologues found in others species, the Hsp 70 protein of *M. tuberculosis* was assayed for phosphorylation (Fig. 3, lane 4). It was incubated in the presence of each of four different metal ions. In each assay, the amount of radioactivity incorporated in the 70 kDa protein was measured and compared to that in a control sample incubated without metal ions. Purified Hsp 70 was significantly labelled *in vitro* in the presence of \([\alpha-32P]ATP\). The capacity of Hsp 70 to phosphorylate under these conditions indicated that it contained an intrinsic protein kinase activity that catalysed its own phosphorylation. The incorporation of labelled phosphate was nearly 300-fold higher in the presence of calcium (7840 vs 28 c.p.m.), 5-fold higher in the presence of manganese (137 c.p.m.) and 2-5-fold higher with magnesium (73 c.p.m.); no significant difference was detected in the presence of zinc ions (32 c.p.m.). Analysis of the acid hydrolysates prepared from autophosphorylated Hsp 70 indicated that phosphothreonine was the only detectable phosphoamino acid, whatever metal ions were present in the incubation medium (Fig. 4b).

**DISCUSSION**

The main result of this study is the demonstration, for the first time, that the small heat-shock protein Hsp 16·3 of *M. tuberculosis* is a phosphoprotein. Also, evidence was obtained that Hsp 16·3 has an intrinsic protein kinase activity which catalyses its own phosphorylation *in vitro*. Moreover, the amino acid residues targeted by this activity were exclusively serine residues, which suggests that Hsp 16·3 is a serine autokinase. Although purified 6His-Hsp 16·3 formed a single band after gel electrophoresis, suggesting that it had been purified to homogeneity, it cannot be excluded that its phosphorylation was due, at least in part, to the activity of a residual co-purified *E. coli* kinase. However, this possibility seems unlikely because no *E. coli* protein kinase has been found to be able to phosphorylate protein substrates from *M. tuberculosis* (Cozzone, 1993).

Autophosphorylation has been demonstrated previously in...
other heat-shock proteins from mycobacteria, including DnaK and GroEL (McCarty & Walker, 1991; Nadeau et al., 1993; Zylicz et al., 1983). This observation was confirmed in our study, which found Hsp 70 can autophosphorylate and thus exhibits functional properties characteristic of the Hsp 70 family. Such functional similarity is further demonstrated by the stimulatory effect of calcium ions on the phosphorylation activity of Hsp 70. The autokinase activity of Hsp 16-3 and Hsp 70 reported here adds to the biochemical similarities between the large and small Hsp families, and suggests that they may share common mechanisms in their mode of action, even though the amino acid residues phosphorylated in Hsp 16-3 (serine) are different from those modified in Hsp 70 (threonine).

Besides these autokinases, other protein kinase activities have been described in mycobacteria. Mycobacterium smegmatis has been reported to contain a soluble calcium/calmodulin-dependent 35 kDa protein kinase with a narrow substrate specificity for both exogenous and endogenous substrates (Sharma et al., 1998). Similarly, M. tuberculosis contains eleven eukaryotic-like serine/threonine protein kinases, the Pkn family, some of which have been characterized in detail (Chaba et al., 2002; Koul et al., 2001). The presence of three phosphoproteins of 50, 55 and 60 kDa, all phosphorylated at tyrosine residues in M. tuberculosis, has also been described, although the enzymic activity responsible for the phosphorylation has not yet been characterized (Chow et al., 1994). Two-component phosphorylation systems are also found in mycobacteria. Several examples of such systems have been reported in M. tuberculosis (Haydel et al., 1999; Mayuri et al., 2002), as well as in M. smegmatis (O’Toole et al., 2003). Together these data indicate that protein phosphorylation is likely to play a crucial role in the metabolism and physiology of mycobacteria (Drews et al., 2001). Blockage of protein kinase activity with specific inhibitors has already been shown to result in limitation of growth (Drews et al., 2001) and a reduction in phagocytosis by macrophages (Prabhakaran et al., 2000).

The phosphoproteins of the Hsp 70 family have been reported to participate in various cellular functions, including binding to unfolded or nascent polypeptides and the renaturation and disaggregation of misfolded polypeptides. Their phosphorylation is known to induce an increased affinity for polypeptide substrates (Peake et al., 1998). These proteins are also involved in replication and protein transport within the cell, and the extent of their phosphorylation is considered an indicator of the level of stress imposed by environmental conditions (Hengge & Bukau, 2003).

In contrast, little is known of the biological role of the phosphorylation of Hsp 16-3. Hsp 16-3 appears to consist of a trimer of trimers with a total molecular mass of 149 kDa (Chang et al., 1996). There may be a relationship between the aggregation of this protein complex and the extent of its phosphorylation. This is seen in several heat-shock proteins, including the α-crystallin protein homologous to Hsp 16-3, in which the disaggregation of the oligomeric structure is accompanied by enhanced phosphorylation (Kato et al., 1994). However, no such relationship was observed after treatment of Hsp 16-3 with the detergent sodium deoxycholate (data not shown).

Hsp 16-3, which is an immunodominant antigen over-produced during the latent stationary phase of M. tuberculosis infection, also seems to act as a chaperone through an ATP-independent process (Groenen et al., 1994; Horwitz, 1992). However, our results from UV cross-linking experiments show that ATP tightly interacts with Hsp 16-3, although it cannot be determined whether the ATP molecules involved in this interaction are those which serve as donors in the phosphorylation reaction. Our results are consistent with a recent report indicating that Hsp 16-3 from M. tuberculosis was protected by ATP against proteolysis by chymotrypsin, whereas no effect was found with a nonhydrolysable analogue of ATP (Valdez et al., 2002), suggesting a close interaction between ATP and Hsp 16-3. However, Hsp 16-3 may bind to other nucleotides, and further studies are needed to evaluate the specificity of the Hsp 16-3/ATP interaction.

The kinase activity of Hsp 16-3 may not be restricted to its own phosphorylation. It may also act on other protein substrates, either within the mycobacterial cell or within the infected cells. It would be interesting to investigate whether there is a phosphoprotein phosphatase activity that can dephosphorylate Hsp 16-3 and/or other substrates. The extent of phosphorylation of Hsp 16-3 should be measured under various physiological conditions in order to evaluate the effect of the environment on modification of this protein.

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REFERENCES


