Mycobacterium tuberculosis 16-kDa Antigen (Hsp16.3) Functions as an Oligomeric Structure in Vitro to Suppress Thermal Aggregation*

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Tuberculosis continues to be a major disease threatening millions of lives worldwide. Several antigens of Mycobacterium tuberculosis, identified by monoclonal antibodies, have been cloned and are being exploited in the development of improved vaccines and diagnostic reagents. We have expressed and purified the 16-kDa antigen, an immunodominant antigen with serodiagnostic value, which has been previously cloned and shown to share low sequence homology with the α-crystallin-related small heat shock protein family. Sedimentation equilibrium analytical ultracentrifugation and dynamic light scattering demonstrate the formation of a specific oligomer, 149 ± 8 kDa, consisting of approximately nine monomers. In 4 M urea, a smaller oligomer of 47 ± 6 kDa (or trimer) is produced. Analysis by electron cryomicroscopy reveals a triangular shaped oligomeric structure arising from the presence of three subparticles or globules. Taken together, the data suggest an antigen complex structure of a trimer of trimers. This antigen, independent of ATP addition, effectively suppresses the thermal aggregation of citrate synthase at 40 °C, indicating that it can function as a molecular chaperone in vitro. A complex between the antigen and heat-denatured citrate synthase can be detected and isolated using high performance liquid chromatography. We propose to rename the 16-kDa antigen Hsp16.3 to be consistent with other members of the small heat shock protein family.

There has been a recent increase in the number of reported cases of tuberculosis (TB), an old scourge and still the leading cause of death among infectious diseases. In addition, multidrug-resistant strains of Mycobacterium tuberculosis, the bacterium that causes TB, have been isolated (for reviews, see Bloom and Murray (1992) and Nowak (1995)). Approximately one-third of the world’s population harbors M. tuberculosis, and the World Health Organization (WHO) predicts that, by the year 2005, TB will kill 4 million people per year (Nowak, 1995). Mycobacteria are daunting organisms to study, and the molecular pathogenesis of TB is still poorly understood (Bloom and Murry, 1992).

In the process of screening for strain-specific antibodies, as well as developing subunit vaccines and efficient diagnostic tests, many murine monoclonal antibodies (mAb)s have been generated against various mycobacterial antigens (Coates et al., 1981; Engers et al., 1986; Ivanyi et al., 1988; J. Acket et al., 1988). Using these mAb s and recombinant DNA techniques, genes of more than a dozen mycobacterial antigens have been cloned (Young et al., 1990; Verbon et al., 1992). Interestingly, several of these major antigens have sequence homologies to the conserved heat shock proteins (Young et al., 1990; Verbon et al., 1992; Kong et al., 1993). Two of these antigens, the 18 kDa antigen from Mycobacterium leprae and the 16-kDa antigen from M. tuberculosis, show sequence homology with the α-crystallin-related small heat shock proteins (Nerland et al., 1988; Verbon et al., 1992). The 16-kDa antigen of M. tuberculosis has previously been referred to as 14K antigen (Engers et al., 1986; J. Acket et al., 1988; Young et al., 1990) and major membrane protein (Lee, et al., 1992) in the literature. This protein is one of the prominent antigens of M. tuberculosis defined by mAb TB68 (Coates et al., 1981), as well as F23-49, and F24-2 (Engers et al., 1986). It carries epitopes restricted to tuberculobacilli (i.e. M. tuberculosis, Mycobacterium africannum, and Mycobacterium bovis) on the basis of B-cell recognition (Coates et al., 1981) and is effective for diagnostic uses (Kingston et al., 1987; J. Acket et al., 1988; Ivanyi et al., 1988; Verstijnen et al., 1989). The cellular location is unknown, although it is proposed to be on the outside of the cell wall (Schonshing et al., 1990; Verstijnen et al., 1989). This antigen, which is capable of generating cell-mediated immune responses, also contains T-cell epitopes which are cross-reactive with M. leprae antigens (Kingston et al., 1987). It is the cell-mediated immune system which plays the dominant role in defense against mycobacteria.

We have previously reported that another immunodominant antigen of M. tuberculosis, the 38-kDa antigen, is a phosphate-binding protein which serves as an initial receptor for active transport (Chang et al., 1994). Here we describe the subcloning, overexpression, and purification of the 16-kDa antigen. Verbon et al. (1992) previously cloned the gene and further noted low level sequence homology with small heat shock proteins (smHsp). We also present direct evidence that the purified recombinant 16-kDa antigen forms a specific oligomer and can function as a molecular chaperone in vitro, consistent with its homology to smHsp.

EXPERIMENTAL PROCEDURES

Materials—HEPES, dithiothreitol, urea, and citrate synthase were obtained from Sigma, and restriction enzymes were from New England Biolabs.

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EXPERIMENTAL PROCEDURES

Materials—HEPES, dithiothreitol, urea, and citrate synthase were obtained from Sigma, and restriction enzymes were from New England Biolabs.
Construction of the Recombinant Expression Plasmid—The 16-kDa antigen was subcloned from pRM144 (kindly provided by Dr. Raju Lathigra, also see Verbon et al., 1992) into the highly efficient pET expression vector of Escherichia coli. The bacterial hosts for cloning and expression was the E. coli K12 strain BL21(DE3) (FompT r6 mB (DE3)) (Studier et al., 1990). The 16-kDa antigen gene was inserted into the pET9d (Novagen, Madison, WI) vector through the cloning with the template being the plasmid pRM144. The 5’ primer is 5’-GCGGATCCACACACCTTCGTTACGGCAACCGC-3’ and the 3’ primer is 5’-GGAGATCCGTGACATCGGCGTGG-3’. NcoI and BamHI restriction sites were engineered into the 5’ and 3’ primers, respectively. PCR was performed using the GeneAmp PCR Reaction Kit (Perkin-Elmer) and the MiniCycler (M) Researcher, according to suppliers’ recommendations. The PCR reaction was performed for a total of 26 cycles. After gel purification, the amplified DNA was cloned into the pET-9d vector following standard procedures (Ausubel et al., 1992) and selected on L-broth/kanamycin plates. Recombinants containing the antigen gene were identified as follows. A few colonies from the selection plate were inoculated in L-broth/kanamycin liquid medium overnight (with no isopropyl-β-D-galactopyranoside inducer), and screened by SDS-PAGE (PhastGel system, Pharmacia Biotech Inc.) for the expression of a dominant protein band at 16 kDa. The recombinant pET-Hsp16.3 plasmid was also isolated from the selected cells and the correct insertion was confirmed by restriction mapping (data not shown).

Protein Expression and Purification—Protein expression from pET-Hsp16.3 in BL21(DE3) was performed following essentially the procedure described by Studier et al. (1990). Cells were grown at 37°C in L-broth medium (plus 40 μg/ml kanamycin) and then induced (at OD600 of 0.8–1) with 0.4 mM isopropyl-β-D-galactopyranoside for 4 h. Cells were harvested by centrifugation, washed with distilled deionized water, and frozen at −20°C until use. Cells, after resuspending in buffer A (50 mM imidazole-HCl, 1 mM β-mercaptoethanol, pH 6.5) with 1 mM AEBSF protease inhibitor (Boehringer Mannheim), were lysed by sonication. The cells from four liters of medium were resuspended in 40 ml of buffer A and sonicated three times, 4 min (W-385 sonicator, Heat system-ultrasonics Inc.). After high speed centrifugation (35,000 × g for 15 min; 30 min), the supernatant was loaded onto a 4 × 36-cm DEAE cellulose column, preequilibrated with buffer A. After washing with about one column volume of buffer A, proteins were eluted with 100 ml total volume of 0–0.3 M NaCl gradient (in buffer A). Fractions containing the 16-kDa protein were located by SDS-PAGE. The fractions containing the 16-kDa protein were pooled (Fig. 1, lane 3) and dialyzed against buffer B (25 mM Tris acetate, 1 mM β-mercaptoethanol, pH 6.5). The dialyzed sample was loaded onto a 1 × 10-cm PEI(NH4) column (J. T. Baker) equilibrated with 200 ml of 0.2 M Tris acetate, pH 6.5, and buffer B. Proteins were eluted with a total of 500 ml buffer B, 1 mM sodium acetate gradient (the 1 mM sodium acetate was made in distilled water with pH adjusted to 6.0).

Protein Sequencing—Amino acid sequencing was performed with an Applied Biosystems 477A protein sequencer in the Protein Sequencing Core Facility of Baylor College of Medicine. Ten residues at the N terminus of the purified recombinant protein (see Fig. 1, lane 4) were sequenced for confirmation.

Gel Filtration—Analytical gel filtration was performed using a 60 × 7.5-mm Bio-Sil 250-10 column (Bio-Rad), connected to a Beckman Optima XL-A analytical ultracentrifuge (Hansen et al., 1994). Runs of three concentrations (0.25, 0.5, and 1 mg/ml) were performed at 10,000 rpm in a Beckman An60Ti rotor at 20°C. Multiple data sets of A280 versus radial position were fit to a single component model using a nonlinear least squares fit with the included software. Dynamic light scattering was performed on a DynaPro-Ray 801 molecular sizing instrument (Protein Solutions, Inc., VA) according to manufacturer’s recommendations.

Electron Cryomicroscopy—A solution of the protein (0.06 mg/ml in 50 mM HEPS, pH 7.0) was frozen in a mixture of vitreous ice using a procedure previously described (Dubochet et al., 1988). The frozen hydrated specimen was kept at −168°C in a Gatan cryoholder and imaged under low dose condition (−6 electrons/Å2) in a JEM 1200 electron cryomicroscope at 100 keV and ×30,000 magnification (Avila-Sakar et al., 1994). The image defocus was aimed at 3–4 μm in order to enhance contrast. Two micrographs were selected for data processing. They were digitized in a Perkin-Elmer microdensitometer with a scanning interval of 16 μm and analyzed with software in IMAGIC (van Heel et al., 1995). 1400 particles were selected from the first micrograph. Each micrograph was band pass filtered and normalized. A reference particle image was generated by rotationally averaging a sum of 100 particle images and was used for translational alignment of the particles in the same micrograph. A circular mask was placed on all aligned particle images, which were then subjected to multivariate statistical analysis (van Heel and Frank, 1981) and automatic classification (van Heel, 1989). Eight classes were used as references for realignment of the original particle images and alignment of an additional 1500 particle images from the second micrograph. The 2900 aligned particle images were subjected to multivariate statistical analysis and classification resulting in 100 classes. 5 images were computed for each class to evaluate the statistical invariance among members in each class (Saiss et al., 1989).

Thermal Aggregation Measurements—The aggregation of CS upon thermal denaturation was determined by measuring the absorption due to increased turbidity from light scattering at 360 nm in a Beckman DU 7500 spectrophotometer with a 12-cell holder accessory. Temperature was controlled using a circulating water bath and measured in the cuvette with a digital thermometer. All experiments were performed in 50 mM HEPS-HCl, pH 7.0, and in a total volume of 200 μl. CS concentration was determined by absorbance at 280 nm, using an extinction coefficient of 1.55 × 10−6 M−1 cm−1 (Singh et al., 1970). Concentration of Hsp16.3 was determined by Bradford (1976) assay.

C5 Thermal Inactivation Experiments—One μl of C5 dimer (87 kDa) was incubated at 39.5–5°C in 1 μM diithiothreitol (DTT). 5 μl of HEPS buffer (200 μl final volume). At the indicated times, 2.6 μl aliquots were removed and assayed for activity at 25°C in 50 mM HEPS, pH 8.1, with a 1-ml total volume, according to Srere et al. (1963), in which production of free CoA was observed after reaction with an excess of 5,5-dithio-bis-(2-nitrobenzoic acid), monitored by an increase in absorbance at 412 nm.

RESULTS

PCR Cloning, Protein Purification, and N-terminal Sequencing—The 16-kDa antigen gene of M. tuberculosis was amplified by PCR cloning from the fusion vector pRM144 and expressed using a pet9d vector in strain BL21 (DE3) (Studier et al., 1990). To facilitate the cloning process a NcoI site and a BamHI site were engineered in the 5′ and 3′ primers, respectively.

The 16-kDa antigen was overexpressed (Fig. 1, lane 2) and purified using two anion exchange columns, DEAE cellulose and PEI(NH)4 silicon. The 16-kDa antigen was eluted at 0.15 M NaCl from the DEAE column and 0.75 M sodium acetate from the PEI(NH)4 column (lanes 3 and 4) and had an approximate isoelectric point of 5.1. The protein was approximately 95% homogenous after these two columns (Fig. 1, lane 4). The av-
The 10 residues at the N terminus of the purified recombinant 16-kDa antigen were determined to be: Ala-Thr-Thr-Leu-Pro-Val-Gln-Arg-His-Pro, confirming the sequence from the gene except that the N-terminal Met residue is missing. Lee et al. (1992) also observed no Met after protein sequencing. We observed similar Met cleavage with another M. tuberculosis antigen (the 38-kDa antigen expressed in the same system) (Chang et al., 1992).

The Recombinant 16-kDa Antigen Forms a Specific Multisubunit Complex—A common feature of the α-crystallin-related smHsp family is the formation of oligomers (for a review, see de Jong et al. (1993)). We also observed a similar feature for the purified recombinant 16-kDa antigen, which shares a low level of sequence homology with the smHsps (Verbon et al., 1992). The apparent molecular mass of the 16-kDa antigen was measured by sedimentation equilibrium analytical ultracentrifugation and dynamic light scattering.

The molecular mass obtained from sedimentation equilibrium was a single species of 151 ± 7 kDa. Dynamic light scattering measurements produced a consistent result of 147 ± 8 kDa. These results indicate the existence of an oligomer of nine subunits. However, the apparent molecular mass estimated from gel filtration is 210 ± 10 kDa. Gel filtration depends on the shape of the protein, and interactions between the protein of interest or standard proteins and the column matrix. In contrast, sedimentation equilibrium provides a more accurate method for the determination of the native molecular weight of a protein, independent of hydrodynamic properties (Laue and Rhodes, 1990).

Electron cryomicroscopic examination of the 16-kDa antigen indicated a triangular shaped particle (Fig. 2A). Further image processing revealed that this shape is due to the presence of three globules or lobes (Fig. 2B). Fig. 2B shows 7 of the class averages and their corresponding S images (see "Experimental Procedures"). The class averages were computed from 2,900 particle images from two micrographs of comparable defocus (~4 μm). These images have been subjected to two rounds of alignment and classification. The S images support the statistical reliability of each of these class averages. Since the protein molecules are presumably oriented randomly on the electron microscopic grid, the class averages represent different projections of the molecule. Fig. 2B shows members of the final class averages with the most obvious trimeric appearance and the least intra-class variance. The edge dimension of the class average with the most apparent 3-fold symmetry is ~100 Å. In the absence of an atomic resolution structure, we cannot yet delineate its quaternary configuration. However, given the evidence that the protein tends to form a 9-subunit complex, these class average images suggest that the 16-kDa oligomer is a trimer of trimers. This oligomeric structure is supported by the finding that the molecular weight of the antigen in 4 M urea or 1 M guanidine HCl was reduced to approximately one-third of the original size (47 ± 6 kDa) as determined by dynamic light scattering. This suggests that the three globules (each a complex of three 16.1-kDa subunits) can be disassembled, and that the total number of subunits in the entire complex is an integral multiple of three. In view of all these observations, we propose that the 16-kDa antigen forms a specific nine-subunit complex with a calculated total mass of 144.9 kDa, based on a monomer mass of 16,100 Da determined by mass spectrometry (Lee et al., 1992).

Inhibition of Thermal Aggregation of Citrate Synthase by 16-kDa Antigen—α-Crystallin and related smHsps have been shown recently to possess molecular chaperone activity (Horwitz, 1992; Jakob et al., 1993; Merck et al., 1993). In light of the sequence homology with smHsps, we tested the antigen for molecular chaperone activity by using pig heart CS, a dimer of identical 43.5-kDa subunits. CS was chosen because it is a commonly used model for folding studies, its thermal aggregation behavior is well characterized, and it has a simple activity assay.

Our results, as shown in Figs. 3, 4, and 5, clearly indicate that the 16-kDa antigen oligomer effectively inhibits the thermal aggregation of CS at 39.5 °C. No suppression of CS aggregation is observed by adding either ribonuclease A or lysozyme under the same assay conditions (Fig. 4). The same holds true for the addition of bovine serum albumin (data not shown). Addition of ATP up to 10 mM with magnesium (up to 1 mM) did not enhance the chaperone activity of the 16-kDa antigen, consistent with other members of the smHsp family.

The 16-kDa antigen also inhibits CS aggregation when added at various times during the incubation of CS alone (Fig. 5). Aggregation is inhibited regardless of how long it was allowed to proceed before antigen addition. Since thermal inactivation of CS is essentially complete after only 10 min (Fig. 6), this suggests that the 16-kDa antigen interacts with a partially unfolded intermediate of CS rather than the native state. Hsp90 was also found to interact with unfolding intermediates of CS at 43 °C (Jakob et al., 1995).

Complex Formation between Heated CS and the 16-kDa An-
Fig. 7 (Panel A) shows the formation of a noncovalent complex (peak 1) between heat-denatured CS and the 16-kDa antigen by HPLC gel filtration. This peak, with a retention time of 10 min, was not present when heated CS or heated antigen were chromatographed alone, nor when a mixture of the two was run without heating. The size of the 10-min peak increased with the time of heating commensurate with a decrease in the peak for the 16-kDa antigen (peak 2) and the peak for CS (peak 3). The 10-min complex peak was collected and aliquots were rechromatographed after various times of incubation at room temperature. The 10-min peak disappeared with a half-life of 56 ± 10 min (first order rate constant = 0.74 ± 0.13 h⁻¹). SDS-PAGE analysis demonstrated that both CS and the 16-kDa antigen were present in the 10-min peak (Fig. 7, Panel B). The ratio between CS and the antigen is approximately 3:1 (weight-to-weight), estimating from the gel band intensity and HPLC peak integration. This gives a molar ratio of 5:1 CS to 16-kDa, based on the assumption that the 16-kDa antigen is a 9-subunit complex and CS is a dimer. However, it is unknown whether the 10-min peak represents one or many species, so this estimate may be unreliable. The accurate molar ratio may be determined by electron cryomicroscopic examination of the complex, a future aim of our studies.

The Enzymatic Activity of CS Is Not Protected by the 16-kDa Antigen—Since the thermal aggregation of CS is effectively suppressed by the 16-kDa antigen (Fig. 3), we further tested whether the enzymatic activity of CS was protected. As shown in Fig. 6, CS loses activity rapidly when heated at 40 °C (Jakob et al., 1995); however, the enzymatic activity of CS is lost to a similar extent with or without the antigen. Shifting the temperature to 25 °C results in detectable renaturation of CS, but this small gain in activity is not affected by the antigen. This evidence also suggests that the 16-kDa antigen interacts with partially unfolded CS, which is enzymatically inactive. Activity assays on the 10-min complex peak isolated by HPLC also showed less than 2% of native CS activity.

DISCUSSION

The small heat shock protein family is the most abundant and diverse group of Hsps, members of which have been dis-
covered in organisms from bacteria to plants to humans. The chaperone activity of smHsps is ATP-independent in contrast to the Hsp60 and Hsp70 families. While the average monomeric molecular mass of smHsps is 15–30 kDa, they usually form 200–800 kDa homo- or hetero-oligomeric complexes (for a review, see de Jong et al., 1993; Jakob and Buchner, 1994, and Arrigo and Landry, 1994), the size of which may be controlled by phosphorylation (Kato et al., 1994).

Our results strongly suggest that the recombinant 16-kDa antigen of M. tuberculosis forms a specific multisubunit complex and can function as a molecular chaperone in vitro in an ATP-independent manner. We will therefore refer to the 16-kDa antigen as Hsp16.3, so named in the Swiss Protein Data Bank by Verbon et al. (accession no. A42651), in order to be consistent with other members of the small heat shock protein family. Recently it has been observed that α-crystallin and a few related smHsps (murine Hsp25, human Hsp27, pea Hsp18.1, and Hsp17.7) can also function as molecular chaperones (Horwich, 1992; Jakob et al., 1993; Merck et al., 1993; Lee et al., 1995). The family of smHsps are much less conserved than the other families of Hsps (Hsp90, Hsp70, and Hsp60) (de Jong et al., 1993; Jakob and Buchner, 1994). The studies presented here provide evidence that the family of small heat shock proteins share common functionality as molecular chaperones. Additionally, preheating Hsp16.3 at 40 °C enhances suppression of CS aggregation (data not shown). The mechanism of this interesting phenomenon is currently being investigated.

Interestingly, Hsp16.3 does not protect citrate synthase from thermal inactivation at 40 °C, although aggregation is completely suppressed (Fig. 3). Hsp18.1 and Hsp17.7 from pea similarly prevented CS aggregation but did not protect CS activity at 45 °C (Lee et al., 1995). We also observed that adding Hsp16.3 into preheated CS halted aggregation (Fig. 5). This strongly suggests, along with the activity data (Fig. 6), that Hsp16.3 binds to partially unfolded intermediates (which are inactive enzymatically) but not native proteins. While this small heat shock protein is effective in suppressing aggregation, other proteins may be needed for the refolding to the native state in vivo (see Jakob and Buchner, 1994).

In light of the molecular weight analysis indicating a mass of approximately 145 kDa for the Hsp16.3 complex, which could be dissociated into trimers in the presence of denaturants, combined with the result of the analysis by electron cryomicroscopy, we conclude that the complex is a trimer of trimers. We are not aware of any other smHsp whose complex structure has been as well defined as that of the Hsp16.3 (Fig. 2B). Indeed, although controversial, several different models of the quaternary structure of α-crystallin have been proposed (for review, see de Jong et al. (1993)). Interestingly, electron microscopic images of negatively stained Hsp17.7 from pea, which is composed of 12 subunits, revealed both round and triangular structures (Lee et al., 1995). If the triangular structure is proven to be similar to the one established for the Hsp16.3, then it is possible that the Hsp17.7 is a trimer of tetramers.

The expression of a homolog of Hsp16.3 in Mycobacterium habana, the 18-kDa antigen, is significantly increased when subjected to heat shock (Lamb et al., 1990). The stress inducibility of Hsp16.3 in M. tuberculosis is not well established and needs to be further investigated (Young and Garbe, 1991).

The size of the Hsp16.3 oligomer under our analytical conditions did not change within the concentration range of 0.4 to 15 mg/ml (data not shown). A similar observation was made with recombinant murine Hsp25 by Behlke et al. (1991). Previous studies suggested that smHsps in mammalian and chicken cells exist in a variety of dynamic states, depending upon the physiological status of the cells (Collier and Schlesinger, 1986; Arrigo et al., 1988). Whether this is the case for Hsp16.3 in M. tuberculosis cells is still unknown.

Lee et al. (1992) found that Hsp16.3 is a major membrane protein, but not likely to be an intrinsic membrane protein judging from its behavior in detergent. In other words, this protein is probably peripherally associated with the membrane. Monoclonal antibody detection did not reveal any secreted Hsp16.3 in culture supernatant (Abou-Zeid, 1988; Verbon et al., 1990). Two smHsps, produced in response to heterologous protein expression in E. coli, were found to be tightly associated with inclusion bodies (Allen et al., 1992). In higher organisms, smHsps were found in the cytoplasm, nuclear and in various organelles (for a review, see Arrigo and Landry (1994)). In vivo location and crystallographic structure analysis of Hsp16.3 are aims for future studies.

Our results demonstrate that the 16-kDa antigen (Hsp16.3)
exists as a specific oligomer, a trimer of trimers, and can function as a molecular chaperone in vitro. Hsp16.3 is easily expressed and provides a good model system for further studies of the function of small heat shock proteins and molecular chaperones in general.

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