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Biochemical and Biophysical Research Communications 305 (2003) 87–93
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Reversible methionine sulfoxidation of *Mycobacterium tuberculosis* small heat shock protein Hsp16.3 and its possible role in scavenging oxidants

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Received 2 April 2003

Abstract

Mycobacterium tuberculosis (TB) small heat shock protein Hsp16.3 was found to be a major membrane protein that is most predominantly expressed under oxidative stress and is localized to the thickened cell envelope. Gene knock-out studies indicate that the Hsp16.3 protein is required for TB to grow in its host macrophage cells. The physiological function of Hsp16.3 has not yet revealed. Our analyses via mass spectrometry, conformation-dependent trypsin digestion, nondenaturing pore gradient electrophoresis, ANS-binding fluorescence measurements, and circular dichroism demonstrate that the three and only the three methionine residues (cysteine and tryptophan residues, which can also be readily oxidized by such oxidant as H_2O_2, are absent in Hsp16.3) can be readily sulfoxidized with H_2O_2 treatment in vitro, and the methionine sulfoxide can be effectively reduced back to the methionine form. Interconversion between the methionine and methioninesulfoxide has been confirmed by selective oxidation and reduction. The sulfoxidation leads to a small degree of conformational change, which in turn results in a significant decrease of the chaperone-like activity. Data presented in this report strongly implicate that reversible sulfoxidation/desulfoxidation of methionine residues may occur in Hsp16.3, which serves as a way to scavenger reactive oxygen or nitrogen species abundantly present in macrophage cells, thus protecting the plasma membrane and other components of *M. tuberculosis* allowing their survival in such bacteriocidal hosts.

Keywords: Small heat shock protein Hsp16.3; *Mycobacterium tuberculosis*; Methionine sulfoxidation; Conformational change; Chaperone activity; Oxidative stress; Oxidant scavenger

Reactive oxygen or nitrogen species in activated macrophage cells are believed to play essential roles to kill the entering bacterial pathogens. However, *Mycobacterium tuberculosis*, the pathogen of tuberculosis, selects macrophage as its host cells. When *M. tuberculosis* cells are grown in culture with the presence of nitric oxide donors (i.e., under oxidative stress), the most predominantly induced protein was found to be Hsp16.3 [1], a small heat shock protein that was originally identified as an immunodominant antigen and can act as molecular chaperones in vitro [2–4]. This protein was found to be a major membrane protein that is most predominantly expressed during the stationary phase of *M. tuberculosis* cultured in vitro and is localized to the thickened cell envelope that appears during this stage [5,6]. The recombinant Hsp16.3 protein was found to exist as a nonamer, forming a specific trimer-of-trimer structure [7,8]. Gene knock-out studies indicate that the Hsp16.3 protein is required for *M. tuberculosis* to grow in macrophages [9]. In addition, two small heat shock proteins (Hsp27 and IbpA/B) were found to be able to protect cells or enzymes from being damaged by reactive oxygen species [10,11]. Although it is generally believed that Hsp16.3 plays an important role in protecting the *M. tuberculosis* cells from being destroyed by the macrophage cells, it is to be revealed on how such protection is realized by the Hsp16.3 protein. A likely

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doi:10.1016/S0006-291X(03)00685-5

scenario is that the Hsp16.3 protein acts to protect the cell membrane from being damaged by the reactive species abundantly present in the activated macrophage cells.

Recent studies have shown that methionine residues exposed on protein surfaces may act as oxidant scavengers, where they are oxidized to form methionine sulfoxide, which can be reduced back to methionine by the action of a specific reductase [12–16]. An apparent question here is whether the methionine residues in Hsp16.3 will act as such oxidant scavengers to protect the *M. tuberculosis* cells in general or its membrane in particular for its survival in macrophage host cells. Interestingly, the Hsp16.3 protein contains three methionine residues, with the Met residue encoded by the initiation codon being removed after translation as previously shown by Chang et al. [7], but no cysteine and tryptophan residues, the two types of residues that are readily modified by reactive oxygen or nitrogen species [14–16].

In this study, the activity of the three Met residues and other residues in Hsp16.3 towards H_2O_2, the reversibility of the modification, effects of such modification on the protein structure, and chaperone-like activity were examined. Our results demonstrate that the three and only the three methionines can be readily sulfoxidized by H_2O_2 treatment at low pH, the methionine sulfoxide can be reduced back to the methionine form, and sulfoxidation leads to a small degree of conformational changes, which in turn results in a significant decrease of the chaperone-like activity. The implication of these observations in terms of the possible physiological function of Hsp16.3 is discussed.

Materials and methods

Materials. Trypsin, α-crystallin, Catalase, and *N*-methylmercaptoacetamide were purchased from Sigma; [^{35}S]methionine (1000 Ci/mmol) was purchased from Amersham. *Escherichia coli* T7 S30 Extract System for Circular DNA (in vitro transcription/translation) Kit was obtained from Promega. All other chemical reagents were of analytical pure.

Plasmid construction, protein expression, and purification. The construction of the expression plasmid vector for Hsp16.3 (pET-Hsp16.3), the overexpression and purification of the recombinant protein from *E. coli* cells were as described previously [7].

Detection of the compactness of oxidized protein conformation via proteolysis. Analysis of the compactness of protein conformation via limited proteolysis was performed according to methods previously described with minor modifications [17]. Briefly, *E. coli* in vitro transcription/translation reaction mixture containing expressed Hsp16.3 protein for 1 h was pretreated at 37 °C for 2 h by hydrogen peroxide at various concentrations and acetone precipitated before subjected to trypsin (with a final concentration of 50 μg/ml) digestion at 37 °C for 1 h. The treated reaction mixture was then applied for SDS–PAGE (15%) and autoradiography analysis. The molecular sizes of the protein forms revealed by the autoradiography bands were estimated by using the Low Molecular Weight Calibration Kit (Pharmacia, Uppsala, Sweden), consisting: phosphorylase *b* (94 kDa), bovine serum albumin (BSA) (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa).

The sulfoxidation and desulfoxidation of Met residues in Hsp16.3. Desalted (using pre-packed RESOURCE RPC from Amersham Phamacia Biotech) recombinant Hsp16.3 protein (1.0 mg/ml) was oxidized at 37 °C for 2 h in the presence of various concentrations of H_2O_2 (prepared in 1% acetic acid), which was then concentrated in Microcon YM-10 (Millipore) via centrifugation, and washed with 1% acetic acid before performing mass spectrometric analysis or with 50 mM PBS (pH 7.0) for other purposes. Reduction (desulfoxidation) of the oxidized sample was carried out in 1.0 M *N*-methylmercaptoacetamide (final concentration) at 37 °C for 12 h, which was then removed via centrifugation, with the proteins washed with 1% acetic acid before applied to mass spectrometric analysis or with 50 mM PBS (pH 7.0) for other purposes. α-Crystallin (1.0 mg/ml) was also treated by using various concentrations of H_2O_2 (prepared in 1% acetic acid) at 37 °C for 2 h, which was then concentrated in Microcon YM-10 (Millipore) via centrifugation, with the proteins washed with 50 mM PBS (pH 7.0) for analysis of chaperone activity.

Mass spectrometric analysis. The oxidized samples were analyzed using PE Sciex API 3000 triple quadrupole mass spectrometer (Thornhill, Ont., Canada) with an electrospray ionization (ESI) interface. A 20 μl sample solution containing 1.0 mg/ml of the oxidized Hsp16.3 protein (prepared according to the above described procedure) was deposited on an ESI probe.

Non-denaturing electrophoresis. Non-denaturing pore gradient polyacrylamide gel electrophoresis was performed to examine the conformational change of oxidized Hsp16.3 protein. The pore gradient gel (5–20%) was prepared in a 125 mm × 100 mm × 1 mm mold and performed according to methods previously described (4 °C, 150 V for 10 h) [18], with the BSA monomer (67 kDa) and dimer (135 kDa) being used as molecular mass standards. Protein bands were visualized by Coomassie blue R-250 staining.

Fluorescence measurements. Fluorescence spectra were measured at 25 °C with a Hitachi F-4000 fluorescence spectrophotometer. To measure the level of ANS binding the emission fluorescence was scanned from 400 to 600 nm with the excitation wavelength set at 390 nm and protein concentration being at 0.1 mg/ml. The final concentration of 8-anilinonaphthalene-1-sulfonate (ANS) present was at 100 μM.

Circular dichroism measurements. Near-UV and far-UV circular dichroism (CD) spectra of Hsp16.3 were measured at 25 °C on a Jasco J-715 spectropolarimeter. The spectra were recorded using a 1 mm path-length cell. The protein concentrations used for near-UV and far-UV CD spectra measurements were at 4 and 0.4 mg/ml, respectively. All the spectra presented were the cumulative average of 15 repeat scans.

Chaperone-like activity assay. The chaperone-like activity of Hsp16.3 was examined by measuring its capacity to suppress the thermal-induced aggregation of catalase at 60 °C, which was monitored at 360 nm on a UV-8500 spectrophotometer. The reaction buffer was 50 mM PBS (pH 7.0); the final concentrations of catalase and Hsp16.3 were 3.0 and 0.6 mg/ml, respectively.

Results

Mass spectrometric analysis of methionine sulfoxidation in Hsp16.3

Methionine residue is known to be specifically oxidized by hydrogen peroxide at low pH [13,19,20]. Oxidation of the methionine residues in Hsp16.3 by H_2O_2 was followed by mass spectrometry, where the increase

of the molecular mass by one or multiple units of 16 Da, representing the addition of one or more oxygen atoms to the molecule [19,21], was monitored. Results presented in Fig. 1 demonstrate that molecular masses of Hsp16.3 with no H_2O_2 treatment (panel A), treated with 2 mM (panel B), 4 mM (panel C), or 8 mM (panel D) H_2O_2, are 16097.0, 16113.2, 16128.6, and 16145.1 Da respectively. Treating with higher concentration of H_2O_2 (up to 100 mM for as long as 72 h) did not result in any further increase of molecular mass (data not shown), suggesting that the three Met residues are the only ones subjected to such oxidation. The apparent mass increments at the three treating concentrations of H_2O_2 (i.e., 2, 4, and 8 mM) were 16, 32, and 48 Da, respectively, reflecting the addition of one, two, and three oxygen atoms to one, two, or three of the three methionine residues at positions of 40, 46, and 69 in Hsp16.3. No cysteine or tryptophan residues (the other two types of residues that can be readily oxidized by H_2O_2) exist in Hsp16.3 and the first Met was found to be removed after translation [7]. Such step-by-step increment of molecular mass of the oxidized Hsp16.3 also indicated a stepwise oxidation mechanism for the three methionines.

Mass spectrometric analysis also showed that the three oxidized forms of Hsp16.3 were all converted back to its non-oxidized form (with the molecular mass being 16097.0 Da) after treating with 1.0 M *N*-methylmercaptoacetamide at 37 °C for 12 h (date not shown). *N*-Methylmercaptoacetamide was demonstrated to be a mild reducing agent that acts specifically on methionine sulfoxide with no adverse action on other residues in peptides or proteins [19,20,22]. Therefore, our results described above strongly indicated the presence of three oxidized methionine residues and ruled out the possibility that the oxidative modification of other amino acid residues could give an increase of 16 Da mass in our experiment condition.

Effect of methionine sulfoxidation on Hsp16.3 structure

What is the consequence for the methionine sulfoxidation in terms of the Hsp16.3 protein structures at the secondary, tertiary, and quaternary levels? Using in vitro translated protein, the effect of the H_2O_2 oxidation on newly synthesized Hsp16.3 structure was assessed by examining the band pattern of the autoradiograph after

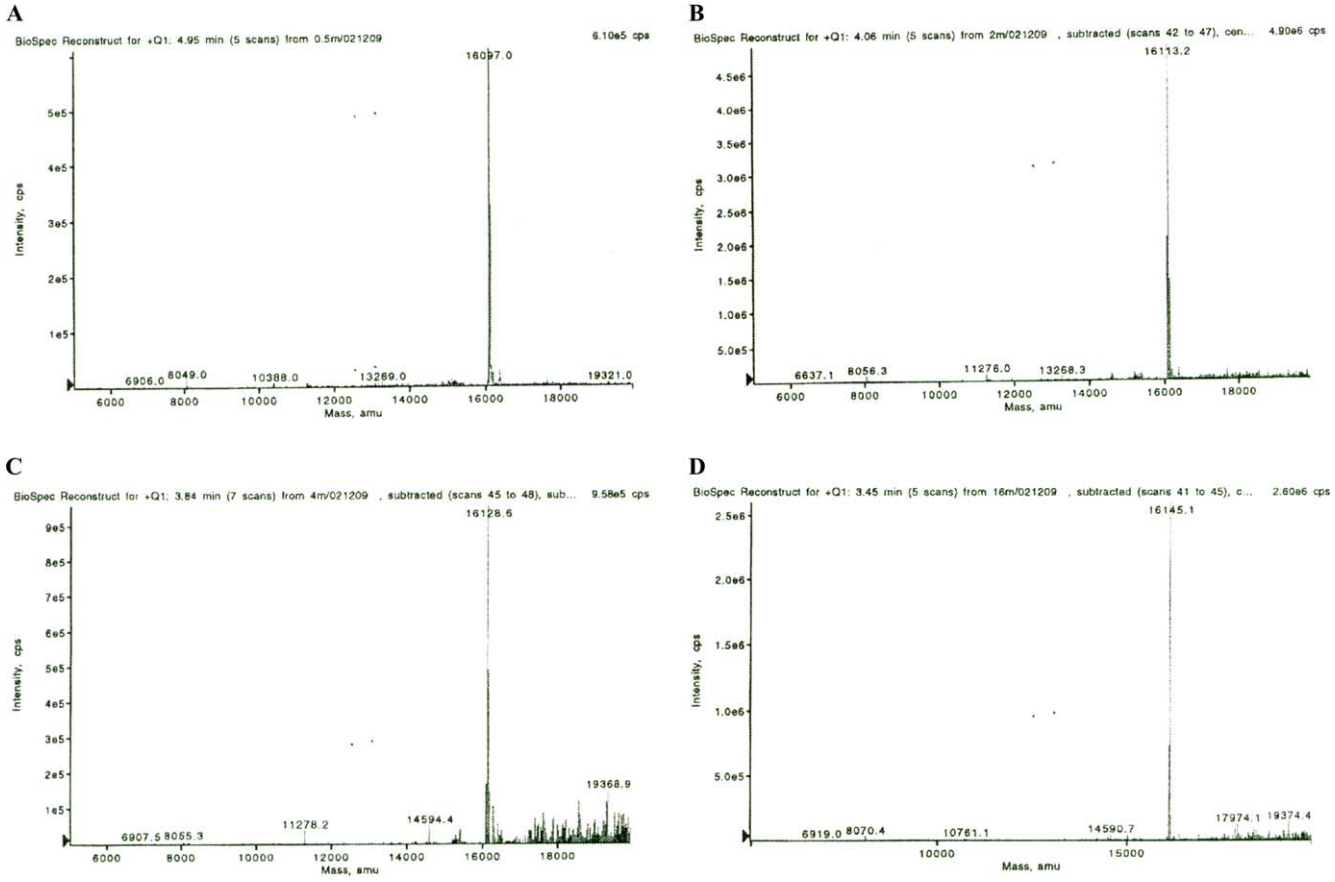

Fig. 1. Mass spectrometric analyses of methionine sulfoxidation in Hsp16.3. LC/MS/MS mass spectra of desalting Hsp16.3 polypeptides are presented. Panels A, B, C, and D correspond to oxidized Hsp16.3 protein treated with 0, 2, 4, or 8 mM H_2O_2 (each at 37 °C for 2 h), respectively.

separating the trypsin digestion products with SDS–PAGE (Fig. 2A). The data demonstrate that Hsp16.3 protein with all three of its methionine residues sulfoxidized is more susceptible to trypsin digestion (lane 4), and those having only one or two Met modified, in contrast, have a similar digestion pattern as the non-treated Hsp16.3 protein (lanes 2, 3, and 1).

The conformation alteration introduced by such methionine sulfoxidation was also analyzed by examining the purified recombinant Hsp16.3 protein with or without H_2O_2 treatment with the non-denaturing pore gradient PAGE (Fig. 2B). Consistent with what has been observed in the trypsin digestion study (Fig. 2A), data presented in Fig. 2B demonstrate that the Hsp16.3 protein with all three methionine residues sulfoxidized (lane 5) migrates significantly faster than those with none, one or two methionine residues modified (lanes 2, 3, and 4, respectively). It should be pointed out that the Hsp16.3 protein appears as a smear when examined through such pore gradient gel electrophoresis due to the dynamic dissociation/reassociation nature of the Hsp16.3 nonamers as demonstrated before [18]. Together, results from both trypsin digestion and non-denaturing pore gradient PAGE strongly indicate that Hsp16.3 with all its three Met residues sulfoxidized exhibits a somehow looser conformation that is more susceptible to protease digestion and having a higher tendency for oligomeric dissociation.

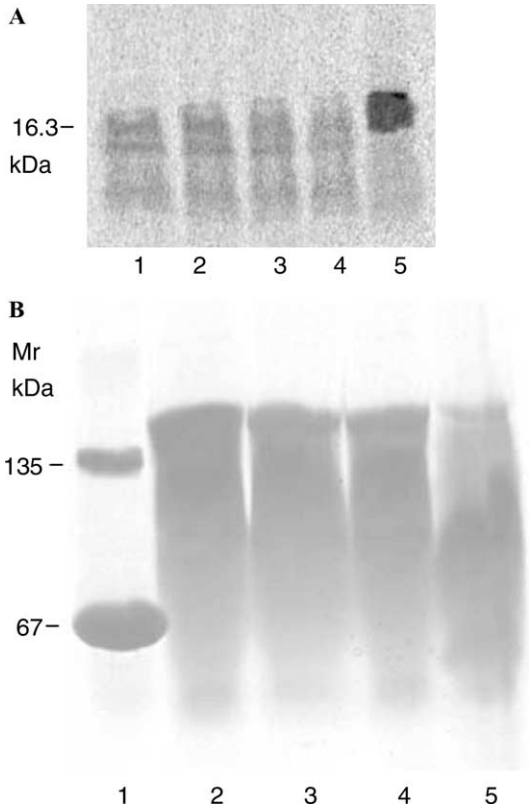

Fig. 2. Examination of the conformational changes of the Hsp16.3 treated with various concentrations of H_2O_2. Shown in panel A is the autoradiograph of the SDS–PAGE (15%) result of the in vitro translated Hsp16.3 after treating with 0 (lane 1), 2 (lane 2), 4 (lane 3), or 8 mM (lane 4) H_2O_2 (at 37 °C for 2 h) and then digested with trypsin for 1 h (with the final concentration of trypsin being at 50 μg/ml). Sample loaded in lane 5, panel A represents the Hsp16.3 protein with neither H_2O_2 treatment nor trypsin digestion. The position where the complete Hsp16.3 protein is expected on the SDS–PAGE is marked on the left of panel A. Shown in panel B is the non-denaturing pore gradient polyacrylamide gel electrophoresis (5–20%) results of the Hsp16.3 treated with 0 (lane 2), 2 (lane 3), 4 (lane 4), or 8 mM (lane 5) H_2O_2 visualized by Coomassie blue staining. All the protein samples were concentrated to 10 mg/ml before applying for the pore gradient PAGE analysis. Molecular standards (BSA monomers and dimers) were loaded in lane 1 of panel B.

The conformational alteration of sulfoxidized Hsp16.3 was also examined by applying ANS to probe the extent of the hydrophobic surfaces of proteins, as well as circular dichroism (CD) spectroscopy to detect structural changes occurring at the secondary and tertiary levels. Results presented in Fig. 3 indicate that the ANS-binding fluorescence spectrum of the Hsp16.3 treated with 2 mM H_2O_2 (second curve from the top) with one Met residue sulfoxidized (see description above) almost overlaps with that of the non-treated protein (top curve). When two or three of the Met residues were modified by sulfoxidation (with the treating concentration of H_2O_2 being 4 and 8 mM, respectively, as described above), the ANS-binding fluorescence spectra appeared at significantly lower positions (curves 3 and 4 from the top, Fig. 3), suggesting a decrease of hydrophobic surfaces in the two modified proteins.

The far-UV CD spectroscopy results (Fig. 4A) reveal that methionine sulfoxidation in Hsp16.3 did not cause any significant alteration at the secondary structure level. The appearance of the spectral minimum at around 217 nm suggests the presence of a high percentage of β-sheets in Hsp16.3 as well demonstrated before [4]. In contrast, near-UV CD spectroscopy results presented in

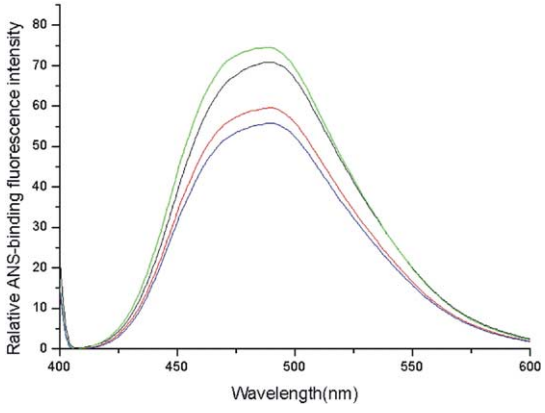

Fig. 3. Fluorescence spectra of ANS bound to oxidized Hsp16.3. Curves from the top to bottom represent the ANS-binding fluorescence spectra, recorded at 25 °C, for oxidized Hsp16.3 samples pretreated with H_2O_2 at concentrations of 0, 2, 4, or 8 mM, respectively.

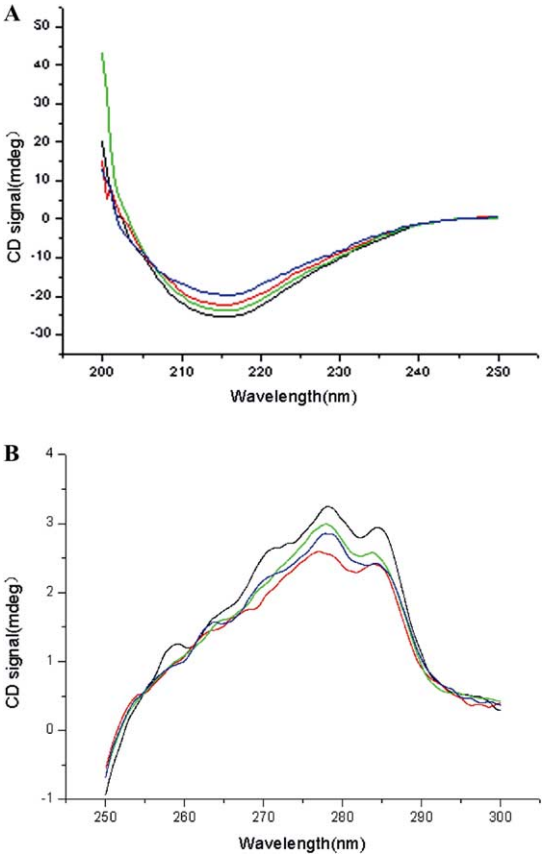

Fig. 4. Far and near UV-CD spectra of oxidized Hsp16.3. Curves in panels A and B represent the far and near UV-CD spectra of oxidized Hsp16.3, respectively. Curves in panel A from bottom to top or in panel B from top to bottom represent the CD spectra of Hsp16.3 pretreated with H_2O_2 at concentration of 0, 2, 4 or 8 mM, respectively.

Fig. 4B reveal that the sulfoxidation of one, two or three Met residues in Hsp16.3 brings in accumulating changes at the tertiary structural level (curves 2, 3, and 4 from the top; with the top curve being the one for non-oxidized protein), as indicated by both the lowering of the peak heights, as well as the shift of the peaks toward the left in the region of 270–290 nm.

Effect of methionine sulfoxidation on the chaperone-like activity of Hsp16.3

Mycobacterium tuberculosis Hsp16.3 acts as a molecular chaperone in vitro [2–4,7,18]. To determine if methionine sulfoxidation affects the chaperone-like activity, the chaperone-like activities (using denaturing catalase as substrate proteins) of Hsp16.3 treated with different concentrations of H_2O_2 (i.e., having either one, two or three Met residues modified) were analyzed, using H_2O_2-treated bovine α-crystallin, also a member of the small heat shock protein family, as a control. Data presented in Fig. 5 indicate that sulfoxidation of one, two or three Met residues in Hsp16.3 (treated with 2.0, 4.0, and 8.0 mM H_2O_2, respectively) increasingly reduces

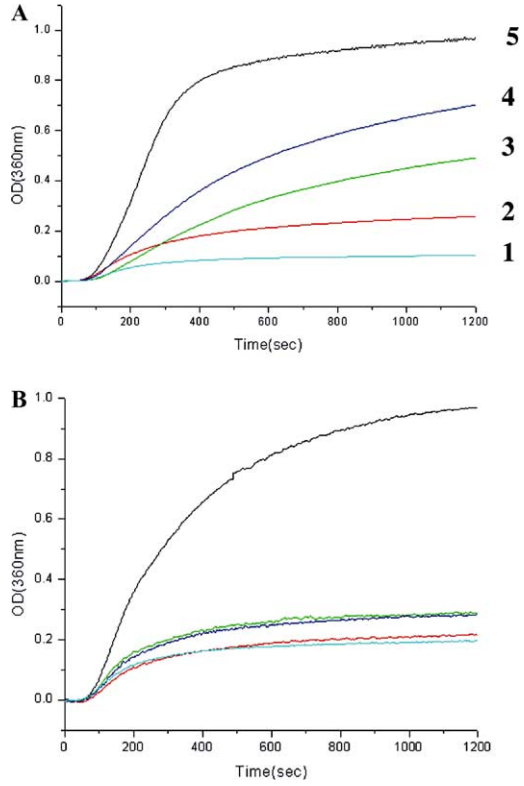

Fig. 5. Chaperone-like activity of Hsp16.3 and α-crystallin with catalase as substrate. Panels A and B are aggregation curves of catalase in the presence of oxidized Hsp16.3 and α-crystallin, respectively. Oxidized Hsp16.3 and α-crystallin were concentrated (10 mg/ml) before applying for the chaperone-like activity analysis. Curves 1, 2, 3, and 4 in panel A represent the Hsp16.3 samples pretreated with H_2O_2 at concentrations of 0, 2, 4, or 8 mM, respectively. The top curve in both panels corresponds to the aggregation curve of catalase with no Hsp16.3 or α-crystallin present.

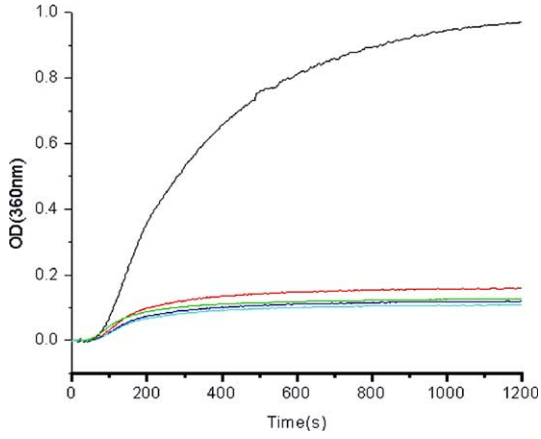

Fig. 6. Chaperone-like activity of Hsp16.3, with denaturing catalase acting as the substrate, after going through a oxidation–reduction cycle. The top curve represents the aggregation process of catalase alone; the bottom four curves represent the aggregation process of catalase in the presence of Hsp16.3 proteins, pretreated with H_2O_2 and then treated with 1 M *N*-methylmercaptoacetamide (37 °C, 12 h). The *N*-methylmercaptoacetamide was removed via centrifugation in Microcon YM-10 (Millipore) and the reduced sample was washed with 50 mM PBS (pH 7.0) and concentrated (10 mg/ml) before applying for this analysis.

the chaperone-like activity (curves 2, 3, and 4 in panel A, respectively), while the chaperone-like activity of α-crystallin was hardly affected by treating with the same concentrations of H_2O_2 (panel B). The chaperone-like activity of Hsp16.3 was almost completely recovered after treating the oxidized Hsp16.3 protein with 1.0 M *N*-methylmercaptoacetamide (with the reducing agent removed by centrifugation in Microcon after the treatment at 37 °C for 12 h) (curves in Fig. 6). This implies that such methionine sulfoxidation process is reversible.

Discussion

Data presented in this report have demonstrated the following properties of Hsp16.3: (1) the three methionine residues in this protein are highly accessible to the non-enzymatic H_2O_2 (and likely other reactive oxygen or nitrogen species) action to form methionine sulfoxides; (2) the methionine sulfoxidation results in an increased tendency of oligomeric dissociation, reduced exposure of hydrophobic surfaces, and altered tertiary structures for the protein, which in turn led to a dramatic reduction in its chaperone-like activity; and (3) the sulfoxides on these methionine residues can be removed by mild reducing chemical reagents (and likely by specific methionine sulfoxide reductase inside the cells), which will recover the chaperone-like activity originally lost by the modification.

Recent studies have led to a belief that specific methionine residues in certain proteins act as oxidant scavengers to protect cells from being damaged by such oxidants like hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), superoxide anion (O_2^-), and reactive nitrogen intermediate (RNI) [14–16]. Protection against such reactive species is apparently essential for the *M. tuberculosis* cells to survive and grow in their hosts, macrophage cells, which are known to utilize a rich source of such reactive species to kill various pathogens endocytosed. Previously studies have clearly shown that the Hsp16.3 protein in *M. tuberculosis* is highly induced when the cell experiences oxidative stress (not induced under heat shock conditions), becomes a major membrane protein after produced, forms a thick layer surrounding the *M. tuberculosis* cell, and significantly reduces the cell's survival capacity in macrophages when its gene is knocked out from the genome [6,9]. An apparent hypothetical role for Hsp16.3 in *M. tuberculosis* would be to protect the pathogen from being attacked by the rich source of reactive oxygen and nitrogen species in the macrophage host cells. The properties we observed here for Hsp16.3 seem to support strongly such a hypothetical role for this protein.

The plentiful existence of Hsp16.3 on the envelope of *M. tuberculosis* cell seems to suggest a role of this protein to protect the non-saturated membrane lipids from being oxidized by such reactive species. It should be pointed out that such non-saturated lipids, especially those with conjugated double bonds, instead of proteins, are the primary targets of the reactive species. The oxidation of the non-saturated lipids would decrease the fluidity of the cell membrane and increase the permeability of the membrane, thus leading to damage or even death of the cells. Needless to say that protection of the cell membrane would be one of the most, if not the most important issue for the *M. tuberculosis* cells to survive in the macrophages. In view of these points, it is likely that exhibition of chaperone activity for Hsp16.3 is not needed, or at least not as important, for the *M. tuberculosis* cells to survive in its host cells. It follows that the reduction or lost of chaperone-like activity for the sulfoxidize Hsp16.3 (as revealed by our study reported here) would not make any problem for understanding the physiological roles of this protein.

If Hsp16.3 indeed acts as oxidant scavenger for *M. tuberculosis* cells, it would be conceivable that the sulfoxidation of the methionine residues is somehow reversible in vivo, which would make possible most likely via the action of the methionine sulfoxide reductase, found to be present in *M. tuberculosis* and essential for protecting the bacteria against oxidative damage from reactive species [23]. Interestingly, reversible methionine sulfoxidation seems to occur for Hsp21, the small heat shock protein present in the chloroplasts of *Arabidopsis thaliana* [21,24,25]. Although no explanation for the physiological role of such post-translational modification was provided, our analysis presented above would implicate that the reversible methionine sulfoxidation of Hsp21 plays a similar role as that of Hsp16.3, i.e., to protect chloroplasts from being damaged by the reactive oxygen species generated during photosynthesis [26].

The susceptibility of the three methionine residues to methionine sulfoxidation by hydrogen peroxide suggests that they are most likely located on the outer surface of Hsp16.3. Given that the protein structure was only slightly affected when one or two of the Met residues were sulfoxidized, moderately affected when all three of them were modified, while both the ANS binding capacity and chaperone-like activity were significantly decreased, it is likely that the three methionine residues somehow are located at sites related to binding denaturing substrate proteins. Our previous observations correlate a higher tendency of oligomeric dissociation with a higher level of chaperone-like activity (Fu et al., unpublished data). However, here the increased tendency of oligomeric dissociation resulted from sulfoxidation of the three Met residues was correlated with a decrease (instead of increase) of hydrophobic surfaces and the chaperone-like activity. A simple explanation for this paradox is that the sulfoxidation converts the hydrophobic side chain of Met into a hydrophilic one (the hydrophobicity index of methionine sulfoxide has

been estimated to be similar to that of lysine, a positively charged residue) [27] at or near the inter-subunit area and/or substrate binding sites, thus decreasing the chaperone-like activity while increasing the tendency of oligomeric dissociation.

In sum, our data provided in this report strongly implicate that reversible sulfoxidation may occur at the three methionine residues of Hsp16.3, which serves as a way to scavenger reactive oxygen or nitrogen species in macrophage cells, thus allowing *M. tuberculosis* to survive in such bacteriocidal hosts.

Acknowledgments

This work was supported by grants from the National Key Basic Research Foundation of China (No. G1999075607), National Science Foundation for Outstanding Young Scientists in China (No. G39725008) (to Dr. Zengyi Chang) and sponsored by the Scientific Foundation for Returned Overseas Chinese Scholars, Ministry of Education (to Dr. Abuduaini Abulimiti). We thank Mr. Chengdui Yang at the Analytical Center of Tsinghua University for technical support and advice concerning the mass spectrometric analysis.

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