Biochemistry (Moscow), Vol. 70, No. 8, 2005, pp. 913-919. Translated from Biokhimiya, Vol. 70, No. 8, 2005, pp. 1104-1111.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-235, February 6, 2005.

Chaperone-Like Activity of *Mycobacterium tuberculosis* Hsp16.3 Does Not Require Its Intact (Native) Structures

Xiaoyou Chen[1], Xinmiao Fu[2,3], Yu Ma[1], and Zengyi Chang[2,3]*

[1]Beijing Tuberculosis and Thoracic Tumor Institute, Beijing 101149, China
[2]State Key Laboratory of Protein Engineering and Plant Genetic Engineering, Peking University, Beijing 100871, China
[3]College of Life Science, Peking University, Beijing 100871, China; fax: 86-10-6275-1526; E-mail: changzy@pku.edu.cn

Received August 14, 2004
Revision received September 25, 2004

Abstract—Small heat shock proteins (sHsps) were found to exhibit efficient chaperone-like activities under stress conditions although their native structures are severely disturbed. Here, using an alternative approach (site-directed mutagenesis), we obtained two structurally and functionally distinct *Mycobacterium tuberculosis* Hsp16.3 single-site mutant proteins. The G59W mutant protein (with Gly59 substituted by Trp) is capable of exhibiting efficient chaperone-like activity even under non-stress conditions although its secondary, tertiary, and quaternary structures are very different from that of the wild type protein. By contrast, the G59A mutant protein (with Gly59 substituted by Ala) resembles with the wild type protein in structure and function. These observations suggest that the Gly59 of the Hsp16.3 protein is critical for its folding and assembly. In particular, we propose that the exhibition of chaperone-like activity for Hsp16.3 does not require its intact (native) structures but requires the disturbance of its native structures (i.e., the native structure-disturbed Hsp16.3 retains its chaperone-like activity or even becomes more active). In addition, the behavior of such an active mutant protein (G59W) also strongly supports our previous suggestion that Hsp16.3 exhibits chaperone-like activity via oligomeric dissociation.

Key words: chaperone, small heat shock protein, Hsp16.3, oligomer, *Mycobacterium tuberculosis*

Small heat shock proteins (sHsps), as one sub-class of molecular chaperones, were found to be present in nearly all types of organisms [1-3]. Like other heat shock proteins, sHsps are induced to high expression level under stress conditions such as heating, osmotic shock, or oxidative conditions, and the over-expression of recombinant sHsps was found to increase the thermal tolerance of organisms [1, 2]. It is reasonably expected that sHsps are able to function as chaperones even at high temperatures at which their native structures are likely disrupted. Indeed both *in vitro* and *in vivo* studies revealed that sHsps are able to bind denaturing substrate proteins and then form tightly bound complexes at heat shock temperatures [4-7]. On the other hand, most sHsps had been found to lose their native secondary, tertiary, and quaternary structures under such heat shock or other stress conditions [8-14]. These observations raise the question of whether the chaperone-like activity of sHsps requires their intact native structures.

To test the above hypothesis, we looked for sHsps that lose the native structures but still maintain the chaperone function by using an alternative approach (site-directed mutagenesis) that is distinct from stress conditions inducing structural disturbance on sHsps. However, sHsps are generally tolerant towards substitutions in primary structure because many mutants that have been reported resembling their wild type counterparts in structure and chaperone function [15-18]. The robust nature of these proteins is thought to be due to their variable quaternary structures [19, 20]. A recent carefully performed mutation study showed that some conserved hydrophobic residues are critical for structure and function of a bacterial sHsp [21], as well as the highly conserved Arg in αA- and αB-crystallin proteins [22-24]. Hsp16.3, originally identified as an immunodominant antigen and later found to be a major membrane protein [25, 26], is the sHsp from *M. tuberculosis* [27]. Previous studies have demonstrated that this protein forms a dynamic nonameric structure using trimers as building blocks [11, 28, 29], and its chaperone-like activity is

Abbreviations: sHsps) small heat shock proteins; G59W) Hsp16.3 mutant protein with Gly59 replaced by Trp; G59A) Hsp16.3 mutant protein with Gly59 replaced by Ala; SEC) size exclusion chromatography.

* To whom correspondence should be addressed.

0006-2979/05/7008-0913

modulated by adjusting the equilibrium and rate of oligomeric dissociation [11, 28, 30, 31].

Here, using Hsp16.3 as a model small heat shock protein, we examined the structural and functional roles of the highly conserved residue Gly59. The substitution of Gly by Trp led to the generation of a mutant protein that has smaller oligomeric size and severely disturbed secondary and tertiary structures but possesses strong chaperone-like activity even at room temperature. Such novel data, in conjunction with previous studies (under stress conditions), suggest that the chaperone-like activity of Hsp16.3 does not require its intact (native) structures. Our studies also strongly support our recent suggestion that sHsps like Hsp16.3 exhibit chaperone-like activity via oligomeric dissociation and the small oligomeric forms are active.

MATERIALS AND METHODS

Materials. Dithiothreitol (DTT), insulin, and bovine serum albumin (BSA) were purchased from Sigma (USA). The MutantBest Kit was purchased from TaKaRa Biotechnology (China). DEAE-Sepharose FastFlow and Q-Sepharose high performance columns for chromatography were all obtained from Amersham Pharmacia Biotech (England). All other chemical reagents were of analytical purity.

Plasmid construction and protein purification. The gene for Hsp16.3 was sub-cloned into the pET-9d expression vector as previously described [27]. Single-site mutated Hsp16.3 (G59A and G59W) were constructed using the MutantBest Kit according a method described in [32]. Recombinant wild type and mutant Hsp16.3 proteins were over-expressed in *E. coli* BL21 (DE3) host cells transformed with the corresponding plasmid. The wild type protein was purified as previously described [11]. To purify the G59W mutant protein, the supernatant of cell lysates was loaded onto DEAE-Sepharose FastFlow column pre-equilibrated with 50 mM imidazole-HCl (pH 6.5) and was then eluted with a salt gradient of 0.12-0.25 M NaCl. The fractions containing G59W were pooled, dialyzed against 20 mM Tris-HCl (pH 8.5), and loaded onto a Q-Sepharose high performance column before being eluted with a salt gradient of 0.15-0.3 M NaCl. The G59A mutant protein was purified similarly with the following modifications: 20 mM Tris-HCl, pH 8.5, was used for the second chromatography operation (elution with 0.15-0.4 M NaCl). The purified proteins were dialyzed in deionized water, lyophilized, and stored at −20°C before further analysis. Protein concentrations were determined by using the Bio-Rad Protein Assay (USA).

Size exclusion chromatography (SEC). SEC was performed on an AKTA FPLC system using a pre-packed Superdex 200 10/30 column (all from Amersham Pharmacia Biotech) at room temperature. For each analysis, a 100 μl protein sample was loaded (centrifuged before loading) and eluted at a flow-rate of 0.4 ml/min with 50 mM sodium phosphate buffer (containing 0.15 M NaCl, pH 7.0). The column was calibrated with the following high molecular mass standards (Bio-Rad): thyroglobulin, 670 kD; bovine gamma globulin, 158 kD; chicken ovalbumin, 44 kD; equine myoglobin, 17 kD; vitamin B_{12}, 1.35 kD.

Circular dichroism (CD) spectroscopy. CD spectra were recorded on a J-715-150L spectropolarimeter (JASCO, Japan). A protein sample of 200 μl (0.2 mg/ml for the far-UV CD and 2 mg/ml for the near-UV CD) was placed in a 2 mm quartz cuvette. The spectra were recorded with 1 nm bandwidth and 0.5 nm step-size at the rate of 1 nm/sec at room temperature. Each spectrum represents the average of four such runs.

Trypsin susceptibility analysis. The wild type or mutant protein Hsp16.3 proteins (1 mg/ml) were subject to trypsin (at 0.005 mg/ml) digestion at room temperature for 30 or 90 min. The trypsin treated proteins were then analyzed using SDS-PAGE (visualized by Coomassie brilliant blue staining).

Fluorescence assay for Trp in the G59W mutant protein. The intrinsic fluorescence of Trp in the Hsp16.3 G59W protein (0.1 mg/ml) was scanned between 300-400 nm while being excited at 279 nm on a F-4500 fluorescence spectrophotometer (Hitachi, Japan).

Non-denaturing pore-gradient polyacrylamide gel electrophoresis (PAGE). The pore-gradient polyacrylamide gel having a 4-20% linear gradient concentration of acrylamide was prepared in a 125 × 100 × 1 mm mold as described [33]. The electrophoresis was performed at constant electric current of 2 mA at room temperature for 18 h and then visualized by Coomassie brilliant blue staining.

Chaperone-like activity assay. Chaperone-like activities of Hsp16.3 were assayed by measuring their capacity to suppress the DTT-induced (20 mM) aggregation of insulin (0.3 mg/ml) at 25 or 45°C, in the presence of the Hsp16.3 protein (0.3 mg/ml of the wild type, G59A, or G59W protein). The aggregation was monitored at 360 nm on a UV-8500 spectrophotometer (Shanghai Techcomp, China).

RESULTS

Substitution of the Gly59 residue of the Hsp16.3 protein with Trp or Ala. The single site-directed mutation on Hsp16.3 were constructed according to the sequence alignments among *M. tuberculosis* Hsp16.3, *M. jannaschii* Hsp16.5, and wheat Hsp16.9, as well as to the crystal structural analysis on the latter two [34, 35]. One loop (amino acids 61-67 in Hsp16.5 (Fig. 1a), as well as the atoms shown as gray ball-and-stick (Fig. 1b)) connecting two anti-parallel β-strands (β3 and β4 in Hsp16.5 (Fig.

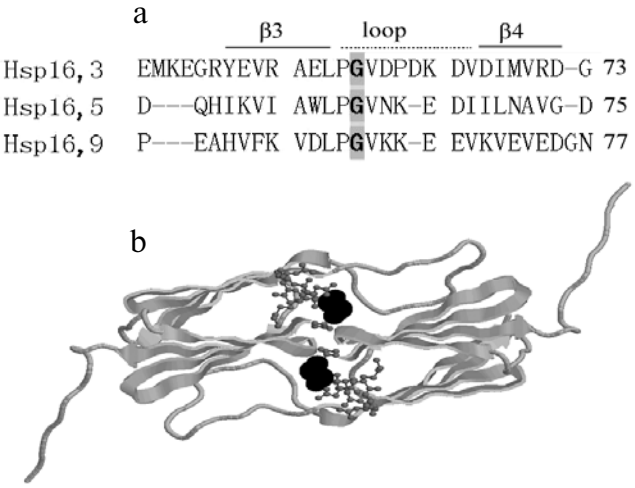

Fig. 1. The structural basis for the Hsp16.3 mutant protein construction. a) Sequence alignment among Hsp16.3, Hsp16.5, and Hsp16.9, only with the loop and the adjacent β-strands shown for simplicity. The mutated Gly59 in Hsp16.3 and the corresponding Gly residues in Hsp16.5 and Hsp16.9 are shown in black. b) The 3-D dimeric structure of Hsp16.5 produced by Raswin (Windows version 2.7.2.1), with the loop shown in gray ball-and-stick model and the Gly62 residue shown in black space-filling model.

1a)) was found to be involved in the dimerization of the protein [34], and it is the same with Hsp16.9 [35]. Of this loop, the residue Gly62 in Hsp16.5 and Gly63 in Hsp16.9 (shown in black in Fig. 1) appear to play important roles in such an oligomerization process through van der Waals forces between the Gly and some hydrophobic residues [34, 35]. In addition, the glycine residue is partially buried (data not shown), in contrast to the subsequent residues (VN/KKED/E, Fig. 1a) in the loop that are located outside of the multimeric oligomers of Hsp16.5 and Hsp16.9 [34, 35]. Based on the above structural analysis and the fact that Hsp16.3 does not contain Trp residues, we constructed a Hsp16.3 mutant protein (G59W) with the corresponding residue Gly59 substituted by Trp, and expected that the large side group in Trp would interfere with the noncovalent forces pre-existing in the protein and thus the folding and/or assembly. To strictly establish the relation between structural perturbation and site-directed mutation, another Hsp16.3 mutant protein (G59A) was also constructed since Ala only has a methyl side group that is much less voluminous than that of Trp.

The secondary, tertiary, and quaternary structures of Hsp16.3 are severely disturbed by the substitution of Gly59 with Trp but not with Ala. We first examined the effect of the substitution of Gly59 on the secondary and tertiary structures of Hsp16.3 protein using far- and near-UV CD spectroscopy, respectively. As presented in Fig. 2a, the far-UV CD spectra of the G59A and wild type Hsp16.3 proteins resemble each other and are dominated by a peak around 215 nm assigned to β-strand (Fig. 2a). The G59W mutant protein appears to primarily retain the elements of β-strand according to the peak near 215 nm; however, a novel peak around 203 nm assigned to random coils [36] was observed (the dotted line in Fig. 2a). This result indicated that the secondary structure of Hsp16.3 is disturbed after the substitution of Gly59 by Trp but not by Ala.

Consistent with the observations on secondary structures, the tertiary structures of the G59W mutant protein appeared to be very different from that of the wild type protein while some but not dramatic changes were

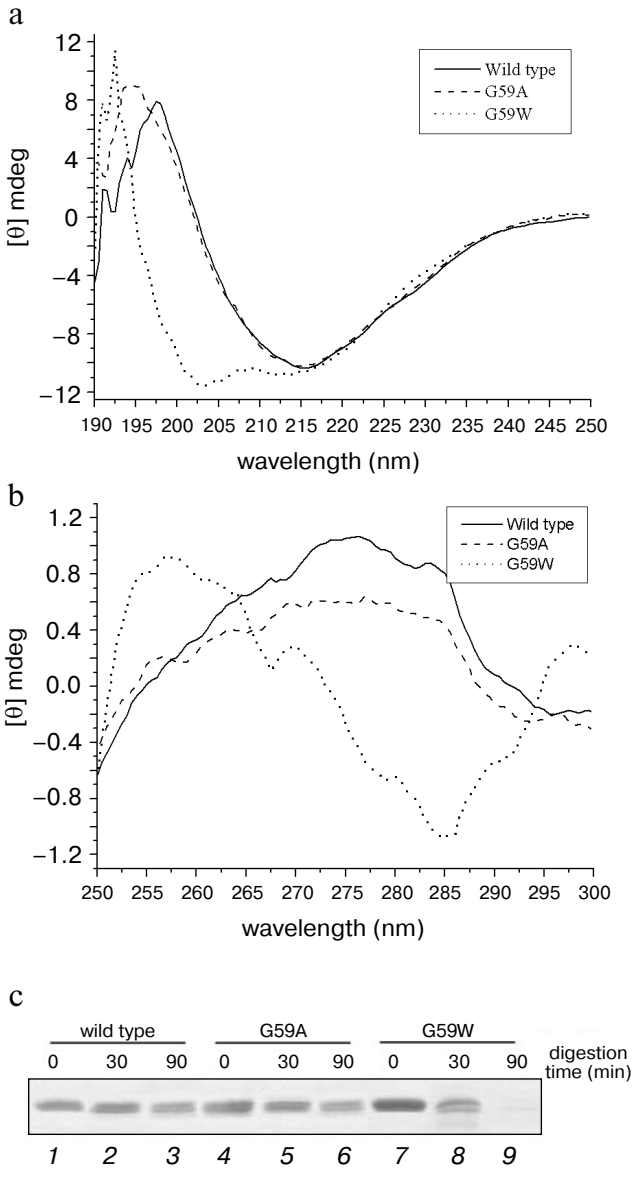

Fig. 2. The effect of substitution on the secondary and tertiary structures of Hsp16.3 protein far-UV (a) and near-UV (b) CD spectra of the wild type, G59W, and G59A Hsp16.3 proteins. The measurement method is described in "Materials and Methods". c) SDS-PAGE analysis results of the wild type, G59A, and G59W Hsp16.3 proteins (1 mg/ml) without or after the digestion by trypsin (0.005 mg/ml) at room temperature for 30 and 90 min.

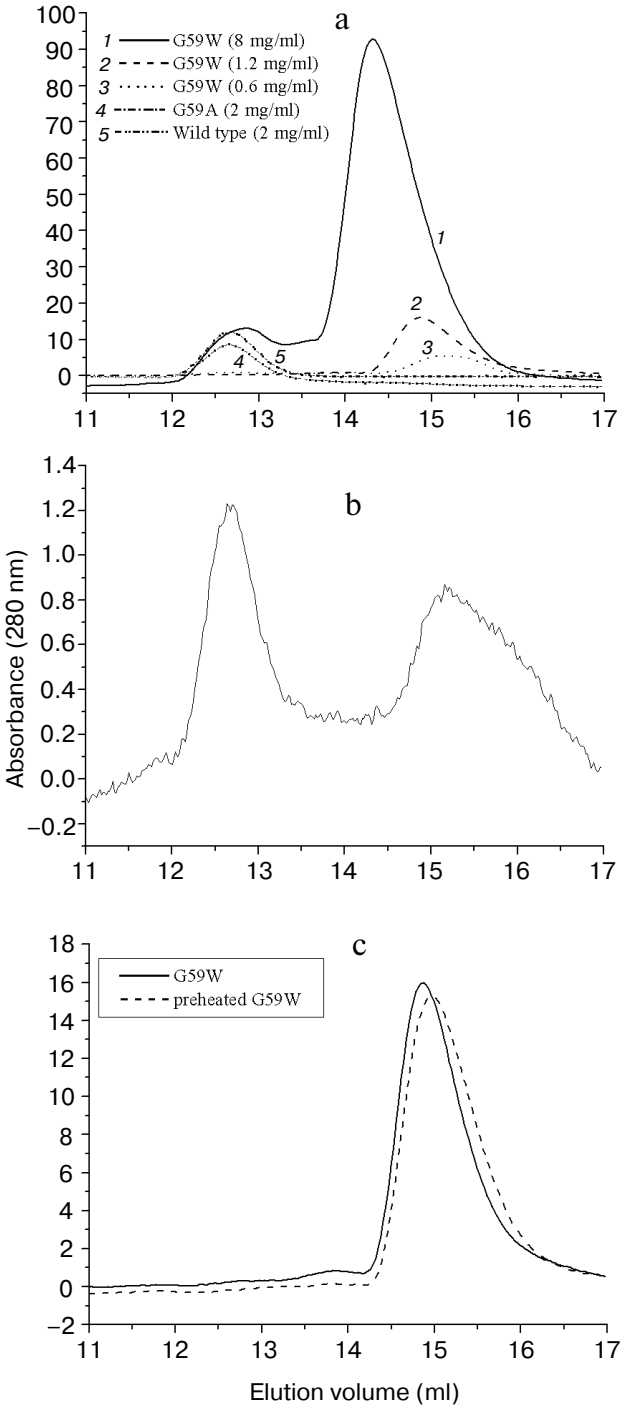

Fig. 3. The effect of substitution on the quaternary structure of Hsp16.3 protein. a) The elution curves of SEC for the wild type (curve *5*), G59W (curves *1-3*), and G59A (curve *4*) Hsp16.3 proteins. b) The elution curve of SEC for the nonameric fraction of the G59W protein collected from the curve *1* in panel (a). The fractions (1 ml) are concentrated into a volume of a little more than 100 μl before chromatography analysis, by centrifugation (at 14,000*g* for 30 min) using a Microcon YM-10 tube (Millipore, USA). c) The elution curves of SEC for the preheated or untreated G59W mutant proteins. The treatment of the protein was performed at 65°C for 15 min and then cooled into room temperature.

observed for the G59A protein (Fig. 2b). These observations indicate that, if the structural roles of Gly59 in Hsp16.3 resemble those in Hsp16.5 and Hsp16.9, the Trp residue might severely interfere with the folding of the protein by spatial hindrance of its large side group, while the Ala residue with a small side group has much weaker effect.

To provide further evidence supporting the significant increase in the element of random coil in the G59W mutant protein, we examined the susceptibilities of the three proteins to digestion by trypsin that preferentially cleaves unfolded protein primarily composed of random coil structures. As shown in Fig. 2c, the wild type and G59A mutant Hsp16.3 proteins were both highly resistant to trypsin digestion within 90 min (comparing lanes *3* and *6* with lanes *1* and *4*, respectively). By contrast, the G59W mutant protein appeared to be significantly cleaved within 30 min (lane *8*), or even completely digested within 90 min (lane *9*).

How does such a substitution on Gly59 affect the oligomeric structure of Hsp16.3 protein? The G59W mutant protein was found to indeed fully exist as small oligomers rather than nonamers, as revealed by size exclusion chromatography (SEC) (curves *2* and *3*, in Fig. 3a). Interestingly, when present at protein concentrations as high as 8 mg/ml, some nonameric forms were detected for the mutant protein (curve *1* in Fig. 3a), indicating that the substitution of Gly59 by Trp just lowers the stability of the nonamers of the Hsp16.3 protein but does not fully disrupt the information for the nonameric assembly. Such an interpretation was supported by the fact that the nonameric forms of the G59W mutant protein, collected from the chromatography fractions shown in Fig. 3a (curve *1*), was able to partially dissociate into small oligomers while re-examined by SEC (Fig. 3b). In addition, the G59A mutant protein retains the nonameric structure like the wild type protein (curves *4* and *5* in Fig. 3a).

Our previous studies have demonstrated that Hsp16.3 [12, 29, 37] as well as other molecular chaperones [38] are able to refold and reassemble after thermally or chemically induced denaturation with high efficiency, and such a property was partially attributed to their high conformation flexibility [28, 32]. The observations on the structural characteristics of the G59W mutant protein raise a question of whether such a structure-disturbed Hsp16.3 protein will still retain its powerful ability to reassemble. Data presented in Fig. 3c show that the thermally denatured mutant protein (at 65°C for 15 min) is indeed able to reassemble into its oligomeric structure after being cooled to room temperature.

The oligomeric structure of the G59W mutant protein is different from that of the wild type protein. The above observations strongly suggest that the Gly59 of Hsp16.3 is a critical residue for both folding and assembly. To further establish this, we examined whether the G59W mutant protein oligomers are compatible with the wild type sub-

units by observing the formation of heterooligomers between these two proteins as a result of subunit exchange, which was found to be a common property for sHsps [11, 39-43].

Two approaches were used. In the first case, neither new peaks (corresponding to the heterooligomers between the G59W and wild type Hsp16.3 proteins) nor changes in the peak height or area of separate proteins were observed, when superimposing the SEC elution curves of the preheated (at 65°C for 15 min) mixture of the two proteins and the separate proteins (Fig. 4a). In addition, the intrinsic fluorescence of Trp in the peak *a* (nonamers) and *b* (small oligomers) for the mixture were found to resemble those for the separate proteins, respectively, thus ruling out the possibility that the heterooligomers were eluted at the same position as the homooligomers of the wild type (peak *a*) or the G59W mutant protein (peak *b*).

In the second case, no heterooligomers between these two proteins were detected when directly visualizing the bands of the mixed proteins on the non-denaturing pore-gradient PAGE (comparing lanes *6* and *7* with lanes *2*, *3*, *4*, and *5* in Fig. 4c). In addition, the electrophoresis behavior of the G59W protein (lane *4*, Fig. 4c) revealed that it predominantly exists as small oligomers besides a minor fraction of nonamers, consistent with that observed in Fig. 3a. Such nonameric forms of the mutant protein appear to be very labile against thermal denaturing since it completely disappeared after being thermally denatured (lane *5*, Fig. 4c).

The G59W mutant protein exhibits much stronger chaperone-like activity than the wild type protein at room temperature. Since the equilibrium or the rate of the oligomeric dissociation process for the Hsp16.3 protein was previously found to modulate its chaperone-like activity [11, 28, 30, 31] and the G59W mutant protein exists predominantly as small oligomers (i.e., the equilibrium of the oligomeric dissociation was fully forward shifted), we examined the chaperone-like activity of this protein. In agreement with our expectation, data presented in Fig. 5 demonstrate that the G59W mutant protein indeed possesses very strong capacity to suppress the DTT-induced aggregation of insulin molecules at 25°C, a temperature at which the wild type protein acts poorly as previously observed [11, 31]. The G59A mutant protein appears to exhibit some but not significant chaperone-like activity. We also observed that these three Hsp16.3 proteins exhibit comparable chaperone-like activities at heat shock temperatures (more than 40°C) (data not shown).

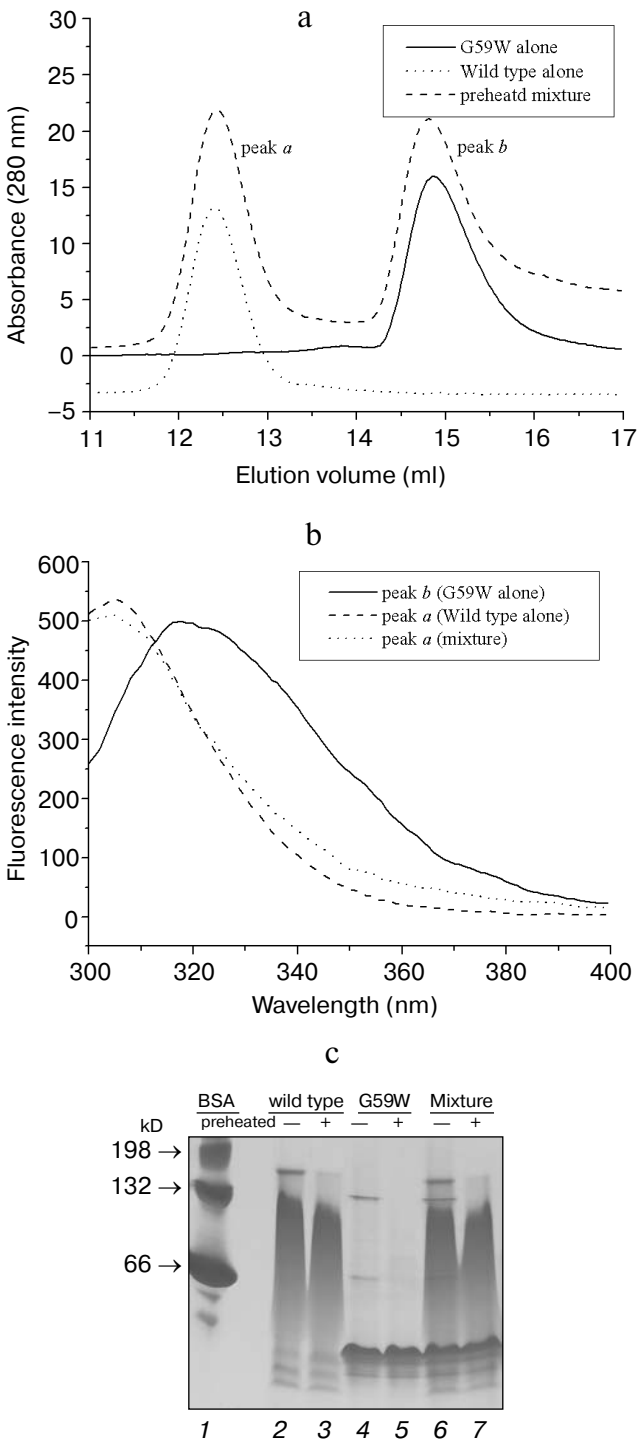

Fig. 4. The G59W mutant protein fails to form heterooligomers with the wild type protein. a) The elution curves of SEC for the wild type, G59W mutant Hsp16.3 protein, and the preheated mixture of these two proteins. Peaks *a* and *b* represent the nonameric and small oligomeric forms of Hsp16.3 proteins, respectively. b) The intrinsic fluorescence of Trp in the fractions (peaks *a* or *b*) collected from panel (a). c) Non-denaturing pore-gradient PAGE analysis results for the wild type protein (lanes *2* and *3*), the G59W mutant protein (lanes *4* and *5*), and the mixture (lanes *6* and *7*). The protein samples were preheated (lanes *3*, *5*, and *7*) or not (lanes *2*, *4*, and *6*) before analysis.

DISCUSSION

Does the chaperone-like activity of Hsp16.3 require the intact secondary, tertiary, and quaternary structures? To our knowledge, the G59W mutant Hsp16.3 protein is

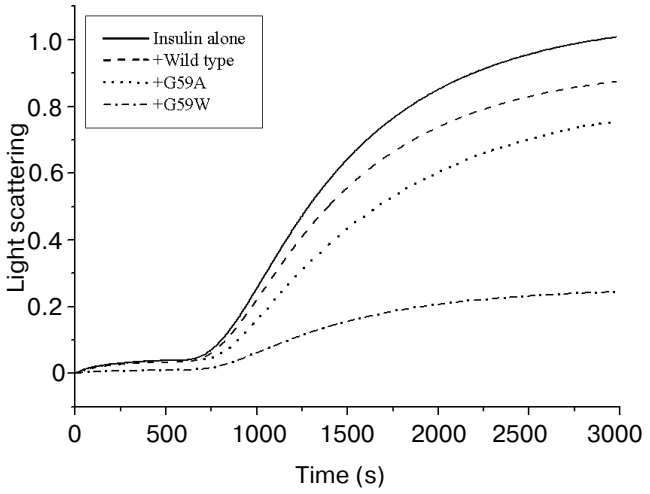

Fig. 5. The effect of substitution on the chaperone-like activity of Hsp16.3 protein. The time-dependent light scattering change recorded at 360 nm for the aggregation of DTT-induced insulin molecules (0.3 mg/ml) performed at 25°C in the absence or presence of the Hsp16.3 proteins (0.3 mg/ml, the wild type, G59A, or G59W protein).

the first example of small heat shock protein with single-site mutation that exhibits efficient chaperone-like activities (at room temperature) while its secondary, tertiary, and quaternary structures are all severely disturbed compared with the wild type protein [15-18, 21, 22]. Hence, such an active mutant protein provides direct evidence to address the question concerning the relationships between structure and function of sHsps. On the other hand, our previous studies revealed that the wild type protein exhibits efficient chaperone-like activities with its secondary, tertiary, and quaternary structures disrupted when exposed to severe stress conditions [11, 12, 32]. In light of these observations, we speculate that the efficient chaperone-like activity of Hsp16.3 does not require the intact (native) secondary, tertiary, and quaternary structures but require the disturbance on its native structures.

Our speculation is supported by the fact that the synthesized 19-residue substrate-binding peptide in α-crystallin indeed exhibits chaperone-like activity [44], assuming that such a peptide is lacking the secondary, tertiary, and quaternary structures as it in the context of the intact protein. As additional supporting data, Hsp26 and α-crystallin were found to function as chaperones with their intact structures disrupted by heating [8-10, 13, 14]. These observations suggest that small heat shock proteins, in contrast to enzymes that are usually labile, exhibit overall conformational flexibility and robust structures in substrate-recognizing and/or binding [32], and their function does not require intact or native structures. Such structural character for sHsps should be considered as a result of the evolutionary selection constraints that require sHsps to efficiently function in the context of stress conditions resulting in the disturbance of the structures of substrate-proteins and sHsps themselves.

The highly conservative residues in Hsp16.3 are critical for the intactness of structure and the accurate modulation of function. Our data that the substitution of Gly59 in Hsp16.3 by Trp but not by Ala led to severe structural disturbance of the mutant protein strongly suggest that this residue plays important roles in the folding and assembly of the protein through van der Waals forces, as implied by the crystal structural analysis on Hsp16.5 and Hsp16.9 [34, 35]. Thus, the implication that the residue corresponding to Gly59 of Hsp16.3 is highly conserved in non-animal sHsps is supported [45].

Our result argues against the generally accepted notion that sHsps are remarkably tolerant towards substitution in primary structure [15-17]. It is also in contrast to some single site mutants on the conserved sites of αA- and αB-crystallin proteins (Arg116 and Arg120 in these two proteins, respectively) that exist as higher or similar molecular complexes but have lower chaperone-like activities [23, 46, 47].

Nevertheless, all these observations indicated that there are still some highly conserved residues that play important structural and functional roles in small heat shock proteins although the overall homology among this protein superfamily was found to be much less than that among the large heat shock proteins such as Hsp70s and Hsp60 [48-50].

This work was funded by the National Key Basic Research Foundation of China (grant Nos. G1999075607 and G1999054104) and the National Natural Science Foundation of China (grant No. 30270289). The authors are grateful to the enlightening discussions provided by members of Dr. Chang's laboratory.

REFERENCES

1. Arrigo, A. P., and Landry, J. (1994) in *The Biology of Heat Shock Proteins and Molecular Chaperones* (Morimoto, R. I., Tissieres, A., and Georgopoulos, C., eds.) Cold Spring Harbor Laboratory Press, New York, pp. 335-374.
2. Buchner, J., Ehrnsperger, M., Gaestel, M., and Walke, S. (1998) *Meth. Enzymol.*, **290**, 339-349.
3. De Jong, W. W., Leunissen, J. A. M., and Voorter, C. E. M. (1993) *Mol. Biol. Evol.*, **10**, 103-126.
4. Lee, G. J., Roseman, A. M., Saibil, H. R., and Vierling, E. (1997) *EMBO J.*, **16**, 659-671.
5. Ehrnsperger, M., Graber, S., Gaestel, M., and Buchner, J. (1997) *EMBO J.*, **16**, 221-229.
6. Friedrich, K. L., Giese, K. C., Buan, N. R., and Vierling, E. (2004) *J. Biol. Chem.*, **279**, 1080-1089.
7. Basha, E., Lee, G. J., Breci, L. A., Hausrath, A. C., Buan, N. R., Giese, K. C., and Vierling, E. (2004) *J. Biol. Chem.*, **279**, 7566-7575.
8. Raman, B., and Rao, C. M. (1994) *J. Biol. Chem.*, **269**, 27266-27268.

9. Raman, B., and Rao, C. M. (1997) *J. Biol. Chem.*, **272**, 23559-23564.
10. Lee, J. S., Satoh, T., Shinoda, H., Samejima, T., Wu, S. H., and Chiou, S. H. (1997) *Biochem. Biophys. Res. Commun.*, **237**, 277-282.
11. Gu, L., Abulimiti, A., Li, W., and Chang Z. (2002) *J. Mol. Biol.*, **319**, 517-526.
12. Mao, Q., Ke, D., Feng, X., and Chang, Z. (2001) *Biochem. Biophys. Res. Commun.*, **284**, 942-947.
13. Haslbeck, M., Walke, S., Stromer, T., Ehrnsperger, M. E., White, H., Chen, S. R., Raibil, H., and Buchner, J. (1999) *EMBO J.*, **18**, 6744-6751.
14. Stromer, T., Fischer, E., Richter, K., Haslbeck, M., and Buchner, J. (2004) *J. Biol. Chem.*, **279**, 11222-11228.
15. Muchowski, P. J., Wu, G. J. S., Liang, J. J. N., Adman, E. T., and Clark, J. I. (1999) *J. Mol. Biol.*, **289**, 397-411.
16. Horwitz, J., Bova, M., Huang, Q. L., Ding, L., Yaron, O., and Lowman, S. (1998) *Int. J. Biol. Macromol.*, **22**, 263-269.
17. Smulders, R. H. P. H., van Boekel, M. A. M., and de Jong, W. W. (1998) *Int. J. Biol. Macromol.*, **22**, 187-196.
18. Berengian, A. R., Parfenova, M., and Mchaourab, H. S. (1999) *J. Biol. Chem.*, **274**, 6305-6314.
19. Haley, D. A., Horwitz, J., and Stewart, P. L. (1998) *J. Mol. Biol.*, **277**, 27-35.
20. Haley, D. A., Bova, M. P., Huang, Q. L., Mchaourab, H. S., and Stewart, P. L. (2000) *J. Mol. Biol.*, **298**, 261-272.
21. Lentze, N., Studer, S., and Narberhaus, F. (2003) *J. Mol. Biol.*, **328**, 927-937.
22. Vicart, P., Caron, A., Guicheney, P., Li, Z., Prevost, M. C., Faure, A., Chateau, D., Chapon, F., Tome, F., Dupret, J. M., Paulin, D., and Fardeau, M. (1998) *Nature Genet.*, **20**, 92-95.
23. Bova, M. P., Yaron, O., Huang, Q., Ding, L., Haley, D. A., Stewart, P. L., and Horwitz, J. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 6137-6142.
24. Litt, M., Kramer, P., LaMorticella, D. M., Murphey, W., Lovrien, E. W., and Weleber, R. G. (1998) *Hum. Mol. Genet.*, **7**, 471-474.
25. Coates, A. R. M., Hewitt, J., Allen, B. W., Ivanyi, J., and Mitchison, D. A. (1981) *Lancet*, **2**, 167-169.
26. Lee, B. Y., Hefta, S. A., and Brennan, P. J. (1992) *Infect. Immun.*, **60**, 2066-2074.
27. Chang, Z., Primm, T. P., Jakana, J., Lee, I. H., Serysheva, I., Chiu, W., Gilbert, H. F., and Quiocho, F. A. (1996) *J. Biol. Chem.*, **271**, 7218-7224.
28. Fu, X., Liu, C., Liu, Y., Feng, X., Gu, L., Chen X., and Chang, Z. (2003) *Biochem. Biophys. Res. Commun.*, **310**, 412-420.
29. Abulimiti, A., Fu, X., Gu, L., Feng, X., and Chang, Z. (2003) *J. Mol. Biol.*, **326**, 1013-1023.
30. Fu, X., Jiao, W., Abulimiti, A., and Chang, Z. (2004) *Biochemistry (Moscow)*, **69**, 552-557.
31. Fu, X., and Chang, Z. (2004) *Biochem. Biophys. Res. Commun.*, **316**, 291-299.
32. Fu, X., Li, W., Mao, Q., and Chang, Z. (2003) *Biochem. Biophys. Res. Commun.*, **308**, 627-635.
33. Yamaoka, T. (1998) *Analyt. Chim. Acta*, **372**, 91-98.
34. Kim, K. K., Kim, R., and Kim, S. H. (1998) *Nature*, **394**, 595-599.
35. Van Montfort, R. L. M., Basha, E., Fieldrich, K. L., and Viering, E. (2001) *Nature Struct. Biol.*, **8**, 1025-1030.
36. Martin, S. R. (1996) *Circular Dichroism*, in *Protein LabFax* (Price, N. C., ed.) Academic Press, pp. 195-204.
37. Feng, X., Huang, S., Fu, X., Abulimiti, A., and Chang, Z. (2002) *Biochem J.*, **363**, 329-334.
38. Ybarra, J., and Horowitz, P. M. (1995) *J. Biol. Chem.*, **270**, 22962-22967.
39. Bova, M. P., Ding, L. L., Horwitz, J., and Fung, B. K.-K. (1997) *J. Biol. Chem.*, **272**, 29511-29517.
40. Bova, M. P., Mchaourab, H. S., Han, Y., and Fung, B. K.-K. (2000) *J. Biol. Chem.*, **275**, 1035-1042.
41. Bova, M. P., Huang, Q., Ding, L., and Horwitz, J. (2002) *J. Biol. Chem.*, **277**, 38468-38475.
42. Sobott, F., Benesch, J. L. P., Vierling, E., and Robinson, C. V. (2002) *J. Biol. Chem.*, **277**, 38921-38929.
43. Studer, S., and Narberhaus, F. (2000) *J. Biol. Chem.*, **275**, 37212-37218.
44. Sharma, K. K., Kumar, R. S., Kumar, G. S., and Quinn, P. T. (2000) *J. Biol. Chem.*, **275**, 3767-3771.
45. De Jong, W. W., Caspers, G. J., and Leunissen, J. A. (1998) *Int. J. Biol. Macromol.*, **22**, 151-162.
46. Andley, U. P., Patel, H. C., and Xi, J. H. (2002) *J. Biol. Chem.*, **277**, 10178-10186.
47. Santhoshkumar, P., and Sharma, K. K. (2001) *J. Biol. Chem.*, **276**, 47094-47099.
48. Narberhaus, F. (2002) *Microbiol. Mol. Biol. Rev.*, **66**, 64-93.
49. Vierling, E. (1991) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **42**, 579-620.
50. Waters, E. R., Lee, G., and Vierling, E. (1996) *J. Exp. Bot.*, **47**, 325-338.