A Dual Role for the N-terminal Region of *Mycobacterium tuberculosis* Hsp16.3 in Self-oligomerization and Binding Denaturing Substrate Proteins*

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The N-terminal regions, which are highly variable in small heat-shock proteins, were found to be structurally disordered in all the 24 subunits of *Methanococcus jannaschii* Hsp16.5 oligomer and half of the 12 subunits of wheat Hsp16.9 oligomer. The structural and functional roles of the corresponding region (potentially disordered) in *Mycobacterium tuberculosis* Hsp16.3, existing as nonamers, were investigated in this work. The data demonstrate that the mutant Hsp16.3 protein with 35 N-terminal residues removed (ΔN35) existed as trimers/dimers rather than as nonamers, failing to bind the hydrophobic probe (1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid) and exhibiting no chaperone-like activity. Nevertheless, another mutant protein with the C-terminal extension (of nine residues) removed, although existing predominantly as dimers, exhibited efficient chaperone-like activity even at room temperatures, indicating that pre-existence as nonamers is not a prerequisite for its chaperone-like activity. Meanwhile, the mutant protein with both the N- and C-terminal ends removed fully exists as a dimer lacking any chaperone-like activity. Furthermore, the N-terminal region alone, either as a synthesized peptide or in fusion protein with glutathione S-transferase, was capable of interacting with denaturing proteins. These observations strongly suggest that the N-terminal region of Hsp16.3 is not only involved in self-oligomerization but also contains the critical site for substrate binding. Such a dual role for the N-terminal region would provide an effective mechanism for the small heat-shock protein to modulate its chaperone-like activity through oligomeric dissociation/reassociation. In addition, this study demonstrated that the wild-type protein was able to form heteronamers with ΔN35 via subunit exchange at a subunit ratio of 2:1. This implies that the 35 N-terminal residues in three of the nine subunits in the wild-type nonamer are not needed for the assembly of nonamers from trimers and are thus probably structurally disordered.

Small heat-shock proteins (sHSPs),1 as one subclass of molecular chaperones, have been found to exist in all types of organisms (1, 2); their expression is inducible in response to elevated temperatures or other stress conditions (3). In vitro studies have demonstrated that sHSPs exhibit chaperone-like activities and are able to prevent the aggregation of denaturing substrate proteins via the formation of tightly bound complexes with them (4, 5). Such complexes are believed to act as a reservoir of the unfolded substrate proteins, which might refold into their native structures with the aid of other types of molecular chaperones (6–8).

Small heat-shock proteins usually exist as oligomers (with 9–40 subunits) that undergo dynamic dissociation/reassociation, a structural feature that is apparently required for their chaperone-like activities (9–15). In the primary structure, they are characterized by the presence of a conserved α-crystallin domain (80–100 residues) that is preceded by an N-terminal region of variable sequence and length and followed by a short C-terminal extension (1, 16, 17). A plethora of studies have demonstrated that the α-crystallin domain is responsible for the formation of dimers that in turn act as building blocks for larger oligomers (12, 18–21), whereas the variation in the N-terminal region of sHSPs is believed to be responsible for their differences in oligomeric size and symmetry (22). truncation of the N-terminal regions of sHSPs from vertebrate (αA-crystallin), *Caenorhabditis elegans* (Hsp16–2), and *Bradyrhizobium japonicum* and *Saccharomyces cerevisiae* (Hsp26) have been reported to lead to the generation of smaller oligomers that failed to exhibit chaperone-like activities (20, 23–26).

One interesting observation is that the N-terminal region was found to be structurally disordered in half of the subunits of the 12-mer of Hsp16.9 (from wheat) and all of the subunits of the 24-mer of Hsp16.5 (from *Methanococcus jannaschii*) as revealed by the determined crystal structures (12, 18). Therefore, two questions were posed with regard to Hsp16.3, a nonameric small heat-shock protein from *Mycobacterium tuberculosis* (27), the three-dimensional structure of which has yet to be determined (28). First, does the N-terminal region in Hsp16.3 help the protein to form its nonameric structure or/and to exhibit chaperone-like activity? Second, does Hsp16.3 share any features with Hsp16.9 or Hsp16.5 in containing disordered N-terminal regions for part or all of the subunits?

Hsp16.3 was originally identified as an immunodominant

dithiothreitol; bis-ANS, 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid; ΔN35, the mutant Hsp16.3 protein with the N-terminal 35 residues truncated; ΔC9, the mutant Hsp16.3 protein with the C-terminal 9 residues truncated; ΔN55ΔC9, the mutant Hsp16.3 protein with the N-terminal 35 residues and C-terminal 9 residues truncated; GST, glutathione S-transferase.

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¶ The abbreviations used are: sHSP, small heat-shock protein; DTT, dithiothreitol; bis-ANS, 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid; ΔN35, the mutant Hsp16.3 protein with the N-terminal 35 residues truncated; ΔC9, the mutant Hsp16.3 protein with the C-terminal 9 residues truncated; ΔN55ΔC9, the mutant Hsp16.3 protein with the N-terminal 35 residues and C-terminal 9 residues truncated; GST, glutathione S-transferase.
antigen and later found to be a major membrane protein (29, 30). Previous studies have demonstrated that Hsp16.3: 1) exists as nonamers, which are assembled by using trimers and hexamers as intermediates (27, 31); 2) undergo dynamic oligomeric dissociation/reassociation (13, 14); and 3) exhibit chaperone-like activities that are modulated by adjusting the equilibrium and/or rate of the oligomeric dissociation process (13–15).

The data presented here conclusively demonstrate that the N-terminal region of Hsp16.3 is not only involved in the formation of nonamers from trimers but also contains the critical site for binding denaturing substrate proteins. The results also suggest that the nonameric structure per se is not a prerequisite for Hsp16.3 to exhibit chaperone-like activities and that the dimeric form is active. In addition, the N-terminal regions in three of the nine subunits of the Hsp16.3 nonamer seem to be structurally disordered. The physiological implications of such a dual role for the N-terminal region in both oligomerization and substrate binding, as well as the existence of disordered N-terminal regions in part of the subunits, is discussed. The Hsp16.3 trimers were suggested to be asymmetric and to be formed by the interactions between a dimer and a third subunit.

**MATERIALS AND METHODS**

**Materials**—Dithiothreitol (DTT), 1,1′-bi(4-amino)naphthalene-5,5′-disulfonic Acid (bis-ANS), and insulin were all obtained from Sigma. MutantBest Kit was purchased from TaKaRa Biotechnology (Dalian, China). DEAE-Sepharose FastFlow and Source Q for chromatography were all obtained from Amersham Biosciences. The N-terminal region peptide of Hsp16.3 (because the first amino acid, Met, is removed in the recombinant Hsp16.3 protein, the peptide of amino acids from 2–35 (i.e. ATTLLFQHRPHSLPFESELFAAFPSFLQGRTF) is synthesized) was provided by GL Biochem Shanghai Ltd. The peptide was synthesized by using the Peptide Synthesizer CS536 (CS Bio Co.), purified by LC-10Avp (Shimadzu, Kyoto, Japan) and identified by the mass spectrometry of Agilent LC-MSD SL. All other chemical reagents were of analytical purity.

**Plasmid Construction and Protein Purification**—The gene for Hsp16.3 was subcloned into the pET-9d expression vector as described previously (27). The Hsp16.3 mutant N35, which is suggested to be the thermally induced formation of nonamers from trimers (28), was constructed using MutantBest Kit, according to the method described previously (32). Recombinant Hsp16.3 wild-type and truncated proteins were over-expressed in *Escherichia coli* strain BL21 (DE3) host cells transformed with the corresponding plasmid.

The wild-type protein was purified as described previously (13). To purify the ΔN35 mutant protein, the supernatant of cell lysates was loaded onto DEAE-Sepharose FastFlow columns pre-equilibrated with 30 mM imidazole-HCl, pH 6.5, and then eluted with a salt gradient made of 0.1–0.4 M NaCl. The fractions containing ΔN35 were pooled, dialyzed against 20 mM Tris-HCl, pH 8.5, and then eluted with a salt gradient made of 0.2–0.5 M NaCl. The ΔC9 protein was purified similarly with the following modifications: 20 mM pipericaine-HCl (pH 5.5; elution, 0.1–0.3 M NaCl) and 20 mM Tris-HCl (pH 8.5; elution, 0.1–0.4 M NaCl) were used for the first and the second chromatography operations, respectively. The ΔN35ΔC9 mutant protein was purified similarly with the following modifications: 20 mM pipericaine-HCl (pH 6.0; elution, 0.1–0.3 M NaCl) and 20 mM pipericaine-HCl (pH 6.0; elution, 0.1–0.3 M NaCl) were used for the first and second chromatography operations, respectively, and subjected to size-exclusion chromatography by Superdex 200 10/30 column.

The gene for the fusion protein of the N-terminal region peptide of Hsp16.3 with glutathione S-transferase (GST) was constructed by using the expression vector pGEX-2T (Amersham Biosciences). The fusion protein of GST-peptide was expressed in the E. coli strain BL21(DE3) at midlog phase of bacterial growth (∆600 of 0.6–0.8) by induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. To purify the fusion protein, the supernatant of cell lysates, suspended in 50 mM sodium phosphate buffer (pH 7.4, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride), was loaded to a glutathione Sepharose 4B affinity matrix. The contaminant proteins were removed by 50 mM sodium phosphate buffer containing 50 mM sodium chloride, and the fusion protein was eluted with 50 mM sodium phosphate buffer containing 10 mM reduced glutathione. The GST protein was also purified with similar procedures. All of 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.

**Chemical Cross-linking by Glutaraldehyde—**Glutaraldehyde (0.5%) and Hsp16.3 proteins reacted at 25 °C in 50 mM phosphate buffer containing 0.15 M NaCl, for 1, 5, and 10 min, as described previously (15). The reaction was stopped by quenching with 1 M Tris-HCl for 10 min. The cross-linked samples were then analyzed through SDS-PAGE (12%) and visualized by Coomasie brilliant blue staining.

**Size-exclusion Chromatography—**Size-exclusion chromatography was performed on a Δ7KTA fast performance liquid chromatography system using pre-packed Superdex 200 10/30 column (all from Amersham Biosciences). For each analysis, a 100-μl protein sample was loaded on a pre-equilibrated column and eluted with a salt gradient made of 300 μl of 0.2 mg/ml was added in a 2-mm quartz cuvette and scanned after being equilibrated at 25 °C for 10 min. The spectra were recorded with a 1-nm bandwidth and a 0.5-nm step size at a rate of 1 nm/s. Each spectrum represents an average of four such runs. Sequence-based prediction of the secondary structural propensity of the N-terminal 35 residues was performed by various programs using different algorithms: 1) PROF, 2) SCRATCH, 3) PHD secondary structure and solvent accessibility prediction, 4) npsupp, 5) HMM-based Protein Sequence Analysis SAM-T99, 6) SOPM, 7) Hierarchical Neural Network, 8) GOR, and 9) PSIPRED. All of the predictions were performed via the links on the web site cn.expasy.org/tools/protscale.html by using the method of Kyte and Doolittle (33). The window size was set to 11 residues.

**Chemical Cross-linking by Glutaraldehyde—**Glutaraldehyde (0.5%) and Hsp16.3 proteins reacted at 25 °C in 50 mM phosphate buffer containing 0.15 M NaCl, for 1, 5, and 10 min, as described previously (15). The reaction was stopped by quenching with 1 M Tris-HCl for 10 min. The cross-linked samples were then analyzed through SDS-PAGE (12%) and visualized by Coomasie brilliant blue staining.

**Non-denaturing Porogradient PAGE—**The porogradient polyacrylamide gel having a 4–20% linear gradient concentration of acrylamide was prepared in a 125 × 100 × 1-mm mold as described previously (34). The electrophoresis was performed at a constant electric current of 2 mA at room temperature for 18 h and then visualized by Coomasie brilliant blue staining.

**Size-exclusion Chromatography—**Size-exclusion chromatography was performed on a Δ7KTA fast performance liquid chromatography system using pre-packed Superdex 200 10/30 column (all from Amersham Biosciences). For each analysis, a 100-μl protein sample was loaded on a pre-equilibrated column and eluted with a salt gradient made of 300 μl.
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Fig. 1. Sequence alignment of the N-terminal regions and the C-terminal extensions for the Hsp16.3, Hsp16.5 and Hsp16.9 proteins. A, sequence alignment of Hsp16.3 with Hsp16.5 and Hsp16.9, with secondary structural elements designated according to Ref. 18, and with α-crystallin domain amino acids not fully shown for simplicity. The structurally disordered N-terminal residues in Hsp16.5 and Hsp16.9 and the corresponding deleted region in the Hsp16.3 protein (ΔN35) are shown in gray, as well as the C-terminal extension residues involved in Hsp16.5 and Hsp16.9 oligomerization and the corresponding deleted residues in the Hsp16.3 protein (ΔC9). B, the hydropathy plot of Hsp16.3 protein. The method of calculation was described under “Materials and Methods.” C, the far-UV CD spectra of wild-type and ΔN35 Hsp16.3 proteins.
To detect the influence of the pre-binding of denaturing substrate proteins on the ability of Hsp16.3 to bind bis-ANS, the wild-type protein (0.2 mg/ml) was pre-incubated with insulin (0.2 or 0.4 mg/ml) and DTT (20 mM) at 25 °C for 5 min before the addition of bis-ANS (final concentration, 100 μM) and UV cross-linking at 65 °C for 20 min. The non-aggregating protein IgG (0.4 mg/ml) was used in place of insulin as a control.

Fluorescence Assay for the bis-ANS Bound to Hsp16.3 Protein—The fluorescence intensity of bis-ANS bound to Hsp16.3 proteins (wild-type and mutant) was measured by scanning between 450 and 560 nm with an excitation wavelength at 390 nm on a Hitachi F-4500 fluorescence spectrophotometer. The Hsp16.3 proteins (0.036 mg/ml) in 50 mM sodium phosphate buffer, pH 7.0, were incubated with 15 μM bis-ANS at 25 °C for 10 min before scanning.

The bis-ANS photoincorporated into Hsp16.3 proteins was analyzed by SDS-PAGE. The gel was first visualized on a UV transilluminator (Biolmage System; UVP, Upland, CA) and later stained with Coomassie brilliant blue.

Subunit Exchange between Wild-type and ΔN35 Mutant Hsp16.3 Proteins—The subunit exchange between these two proteins was examined by size-exclusion chromatography. In brief, the purified wild-type and ΔN35 proteins were mixed at different mass ratios (0.5:0, 0.1:0.5, 1:1, 2:1, or 2:1:0.5, where 1 represents 2.4 mg/ml) and incubated at room temperature for 24 h before size-exclusion chromatography analysis. The protein samples pooled at each elution peak were concentrated with a MicroconYM-3 tube (Millipore) before being examined by SDS-PAGE (visualized by Coomassie brilliant blue staining). The protein bands on the gel were quantitated by densitometric analysis, using the ImageMaster total lab software (version 1.0; Amersham Biosciences).

Native PAGE—The blue native PAGE having a 4–30% linear gradient concentration of acrylamide was prepared as described previously (36). The electrophoresis was performed at a constant electric current of 6 mA at 4 °C. The gel was stained with Coomassie brilliant blue.

**RESULTS**

The Removal of the Potentially Disordered N-terminal 35 Residues of Hsp16.3 Had Little Effect on the Protein Secondary Structure—Sequence alignment of the whole sequences of Hsp16.3, Hsp16.5, and Hsp16.9 (Fig. 1A) revealed that the 35 N-terminal residues of Hsp16.3 correspond to the N-terminal regions of Hsp16.9 and Hsp16.5, which are structurally disordered in half of the 12 subunits of the former and all 24 subunits of the latter (12, 18). To probe the structural and functional roles of the corresponding region in Hsp16.3, a mutant protein (ΔN35) with this region removed was generated. Far-UV CD spectroscopy was first applied to examine whether the secondary structure of the protein is affected by such truncation. The result shown in Fig. 1C indicates that the secondary structure of Hsp16.3 was almost unaffected by the removal of this N-terminal region, and β-sheets were dominant in both the wild-type and mutant Hsp16.3 proteins, as indicated by the peak at around 215 nm. This result indicates that the remaining amino acids retained the primarily β-sheet structure of the whole protein. Supporting this hypothesis is the secondary structure prediction result, which suggests that the N-terminal 35 residues of Hsp16.3 most likely exist as random coils (data not shown).

The ΔN35 Mutant Hsp16.3 Protein Exists As Trimmers/Dimers Rather Than Nonamers—How does the removal of the N-terminal 35 residues affect the oligomeric size of the Hsp16.3 protein? Analysis results using three various methods (all presented in Fig. 2) unequivocally demonstrate that the ΔN35 mutant protein exists as trimers/dimers rather than nonamers. In the first place, only covalently linked trimers and dimers were detected when the mutant protein was cross-linked by glutaraldehyde (lane 9 in Fig. 2A). In the second place, only bands corresponding to trimers and dimers were observed when the mutant protein was analyzed by using non-denaturing pore-gradient PAGE (Fig. 2B, lane 3). Finally, the ΔN35 mutant protein was eluted in size-exclusion chromatography analysis as two overlapping peaks corresponding to its trimer and dimer forms when loaded at high protein concentrations (Fig. 2C, curve 4), as one peak of its dimer form when loaded at low protein concentrations (curve 7), and again as trimer and dimer when the diluted protein was recondensed before chromatography (curve 8). This result indicated that the trimeric form of the ΔN35 mutant protein is in a concentration-dependent equilibrium with the dimeric form. The presence of a high level of monomers in the mutant protein samples subject to chemical cross-linking (Fig. 2A, compare lanes 8 and 9 with lanes 4 and 5) may indicate a weaker interaction between the subunits in the trimers/dimers, allowing modification by glutaraldehyde but also allowing the subunits not cross-linked to dissociate and exist as monomers.

The ΔN35 Mutant Hsp16.3 Protein Fails to Exhibit Chaperone-like Activities—We then examined whether the removal of the 35 N-terminal residues would affect the chaperone-like activity of Hsp16.3. A complete lack of such chaperone-like activity for the ΔN35 mutant protein, as indicated by its failure to suppress DTI-induced aggregation of insulin proteins at 45 °C, is unambiguously shown by the results presented in Fig. 3. A similar result was also obtained when the whole-cell extracts of *E. coli* were used as the substrate, whereas the wild-type Hsp16.3 effectively suppressed the thermal aggregation of such whole-cell extracts (data not shown). Consistent with these observations, no complexes between the ΔN35 mutant protein and denaturing insulin molecules were detected by size-exclusion chromatography (Fig. 4C).

### The Hsp16.3 Protein with Nine Residues from C-terminal-truncated (ΔC9) Exists Mainly As Dimers and Exhibits Efficient Chaperone-like Activities Even at Low Temperatures

The lack of chaperone-like activity for the ΔN35 mutant Hsp16.3 protein is caused by either the failure of the mutant protein to form nonamers (20, 21, 37, 38) or the direct involvement of the N-terminal region in binding denaturing substrate proteins.

To test the first possibility, an Hsp16.3 mutant having the N-terminal region intact but unable to form nonamers would be needed. In this connection, it was noted that the C-terminal extensions of Hsp16.5 and Hsp16.9 apparently contribute to the formation of large oligomers as revealed by their crystal structures (12, 18). Nine residues from the C-terminal of Hsp16.3 (see Fig. 1A) were thus removed. Using size-exclusion chromatography (Fig. 4A) and chemical cross-linking (Fig. 4A, inset), the mutant protein (ΔC9) was found to indeed exist mainly as dimers in addition to a minor fraction of nonamers. In contrast to the behavior of the trimeric/dimeric ΔN35 protein, the dimeric ΔC9 protein was able to suppress the aggregation of insulin at 45 °C with efficiency comparable with that of the wild-type Hsp16.3 proteins (Fig. 3). More interestingly, the ΔC9 protein acted efficiently in suppressing the aggregation of denaturing insulin molecules at 25 °C, at which temperature the wild-type protein has a markedly low effectiveness (Fig. 4B). Consistent with the activity assays, the ΔC9 mutant protein was found to be able to form complexes with denaturing insulin molecules (Fig. 4C).

### The N-terminal Region in Hsp16.3 Is Involved in Binding Denaturing Proteins

Taken together, the results described above strongly imply that the N-terminal region of the Hsp16.3 protein directly participates in binding denaturing substrate proteins. To provide additional evidence to support this contention, the availability of hydrophobic surfaces, needed for substrate binding, was examined for the ΔN35 mutant protein by using a commonly used hydrophobic probe, bis-ANS (6, 35, 39). The results presented in Fig. 5 clearly demonstrate a lack of binding of the probe to the ΔN35 mutant protein: the expected increase of fluorescence of bis-ANS was not recorded when it was mixed with the mutant protein (A), and the photoincorporation of the bis-
ANS into the mutant protein was not detected (B).
The overlapping nature of the sites for both hydrophobic probe binding and substrate binding in the wild-type Hsp16.3 protein was determined by showing the efficient reciprocal suppression between the two processes (Fig. 6): either the chaperone-like activity of the wild-type protein was greatly decreased after the pre-binding of bis-ANS (Fig. 6A) while maintaining its nonameric size unchanged (data not shown) or the amount of bis-ANS photoincorporated into the wild-type protein was also decreased after the pre-binding of denaturing insulin molecules to the protein (Fig. 6B, lanes 3 and 4 versus lane 1). These observations strongly suggest that the N-terminal 35 residues of Hsp16.3 are involved in binding the hydrophobic probe and in turn the denaturing substrate proteins.
The N-Terminal Region of Hsp16.3 Either As Synthesized Peptide or in Fusion with GST Enhances the Aggregation of Denaturing Substrate Proteins—Therefore, it is essential to determine whether the N-terminal region of Hsp16.3 alone is adequate to exhibit chaperone-like activity. Two investigative methods were used. First, the N-terminal region was directly synthesized as a free peptide of 34 residues (see “Materials and Methods”). The results shown in Fig. 7A persuasively demonstrate that such a peptide is able to dramatically enhance the DTT-induced aggregation of insulin at 25 °C (compare curve 2 with curve 1). Such an enhancing effect is readily apparent to the unaided eye; the reaction mixture of DTT and the peptide become turbid as an immediate consequence of the addition of insulin. No aggregation was observed for the mixture containing the peptide and DTT (Fig. 7A, first half of curve 2) and no enhancement effect when IgG was used instead of the peptide (curve 4). Although aggregation was not detected at all in the mixture of the peptide and insulin (Fig. 7A, first half of curve 3), immediate and rapid aggregation occurred right after the denaturant DTT was added into the mixture (Fig. 7A, recorded by the second half of curve 3). A similar (but weaker) effect was also detected when insulin was substituted with a larger substrate protein, lysozyme (Fig. 7B). All these observations indicate that the N-terminal region peptide is capable of interacting with denaturing insulin B molecules, reflecting its ability to bind denaturing substrate proteins in the context of Hsp16.3 protein.

Second, the N-terminal region of Hsp16.3 was produced as a fusion protein attached to the C-terminal end of GST (see “Materials and Methods”). The fusion protein was found to significantly enhance the DTT-induced aggregation of insulin molecules at 37 °C, as represented by curve 2 (compared with curve 1) in Fig. 7C, whereas the GST protein had a limited but insignificant effect on such a process (curve 3). It is noteworthy that this enhancing effect was undetectable when the aggregation was monitored at 25 °C (data not shown).

To better understand the molecular processes occurring during the aggregation reactions described above (with data presented in Fig. 7A–C), the aggregate formed in each reaction mixture was collected separately by centrifugation and examined together via SDS-PAGE, with the results presented in Fig. 7D. It is unmistakably apparent that the peptide was found in the aggregates of lysozyme (lanes 5 and 6), and the fusion protein was found in the aggregates of insulin (lane 8), whereas the GST protein alone was marginally detectable (lane 9). Because of the closely comparable molecular size of the peptide and insulin B chain (3.8 and 3.4 kDa, respectively), the presence of the peptide in the insulin aggregate was not readily apparent; rather, it was strongly suggested by the higher den-
sity of the protein bands representing a co-migration of the two (Fig. 7D, compare lanes 2 and 3 with lane 1). It was also noted that the amount of the peptide or the fusion protein seemed to be much less than that of the substrate proteins in the aggregates (lanes 5, 6, and 8), suggesting that the molecular ratio between the peptide/fusion protein and substrate protein in the aggregate was less than one.

Taken together, these observations are in accordance with the conclusion that the N-terminal region of Hsp16.3 in the context of the whole protein is a critical site for substrate binding.

**Heterononamers of Fixed Subunit Ratio Are Formed between the Wild-type and ΔN35 Hsp16.3 Proteins**—The data presented in Fig. 2, indicating the importance of the N-terminal region in protein oligomerization, ruled out the scenario in which Hsp16.3 has a disordered structure for the N-terminal regions in all the subunits (as was the case for Hsp16.5 (18)). Would the Hsp16.3 protein share with Hsp16.9 the disordered structure in part of the subunits (12)? A conclusive answer to this question remains unattainable because of the lack of success in determining its three-dimensional structure using x-ray crystallography (28). Considering that the disordered N-terminal regions of Hsp16.5 are not needed for its oligomeric assembly (18, 40), we attempted to answer this question via an indirect approach in accordance with the following logic. If the N-terminal region of Hsp16.3 is disordered in part of the nine subunits and thus not needed for the formation of nonamers, the ΔN35 protein subunit would be able to substitute the subunits of the wild-type protein having a disordered N-terminal region (through subunit exchange, as demonstrated by prior investigation (13, 31)), thus generating heterononamers.

The results presented in Fig. 8 unmistakably clearly demonstrate the formation of such heterononamers. First, the incorporation of the ΔN35 mutant protein into the nonamers, when a fixed amount of mutant trimers/dimers were mixed with an increasing amount of the wild-type Hsp16.3 nonamers, is indicated by the proportional decrease of the trimer/dimer peak (Fig. 8A, peaks a) accompanied by a concomitant increase of the nonamer peak (Fig. 8A, peaks c) in the size-exclusion chromatography elution curves. Second, both wild-type and ΔN35 mutant proteins were detected in the fractions from the nonamer peaks using SDS-PAGE (Fig. 8B, lanes 3, 5, 7, and 9), proving unequivocally the formation of heterononamers. It is interesting that the heterononamers were found to be formed with a fixed 2:1 subunit ratio between the wild-type and the
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FIG. 7. The synthesized peptide of N-terminal region and its fusion protein with GST are able to interact with denaturing proteins. A, the time-dependent change of light scattering (360 nm) for DTT-induced (20 mM) aggregation of insulin (0.4 mg/ml) at 25 °C in the presence of synthesized N-terminal region peptide (0.4 mg/ml; curves 2 and 3). IgG was used as a control (curve 4). For curve 2, insulin was added at the time of 800 s after the peptide and DTT were mixed, as shown by the left arrow; for curve 3, DTT was added at the time of 1000 s after insulin and the peptide were mixed, as shown by the right arrow. B, the time-dependent change of light scattering (360 nm) for DTT-induced (20 mM) aggregation of lysozyme (1 mg/ml) at 35 °C in the absence (curve 1) or in the presence of synthesized N-terminal region peptide (0.4 mg/ml; curves 2 and 3). For curve 2, DTT was added at the time of 1000 s after insulin and the peptide were mixed, as shown by the arrow. C, the time-dependent change of light scattering (360 nm) for DTT-induced (20 mM) aggregation of insulin (0.4 mg/ml) at 37 °C in the absence or in the presence of synthesized N-terminal region peptide (0.4 mg/ml; curves 2 and 3). For curve 2, DTT was added at the time of 520 s after insulin and the peptide were mixed, as shown by the arrow. D, SDS-PAGE analysis results for the aggregate formed in each reaction mixture as described in A, B, and C. Lanes 1, 2, and 3, aggregates of the reaction mixtures of curves 1, 2, and 3, respectively, of A; lanes 4, 5, and 6, curves 1, 2, and 3, respectively, of B; and lanes 7, 8, and 9, curves 1, 2, and 3, respectively, of C.

mutant proteins as revealed by densitometric analysis of the two protein bands (the measured weight and subunit ratios between the wild-type and mutant forms are shown in Fig. 8B below lanes 3, 5, 7, and 9). The validity of the 2:1 ratio is confirmed by the almost complete incorporation of the mutant protein into the wild-type nonamer when the wild-type and mutant Hsp16.3 proteins were mixed with a subunit ratio of 2 to 1 (corresponding to a mass ratio of 2:1.0:8; see Fig. 8A, curve 7). These observations strongly suggest that the N-terminal 35 residues in three of the nine subunits in wild-type Hsp16.3 nonamer are not needed for the assembly of nonamers from trimers and are thus most likely disordered in structure. Meanwhile, such a subunit ratio of 2 to 1 seems to be fully consistent with previous proposals that Hsp16.3 uses trimers, instead of dimers, as the basic assembly unit (27).

An additional interesting observation is that heterotrimeric/dimeric containing both the wild-type and ΔN35 subunits were not detected by the SDS-PAGE analysis of samples collected from the trimer/dimer peaks (i.e. Fig. 8A, peaks b), where only the ΔN35 mutant form but no wild-type protein was detected (Fig. 8B, lanes 4, 6, 8, and 10). This phenomenon may be explained as follows: either the heterotrimeric/dimers were formed but immediately assembled into the more stable heterononamers (which contain one ΔN35 subunit in each trimer) or, in a much less likely scenario, the heterononamers are assembled from one ΔN35 homotrimer and two wild-type homotrimers.

The Hsp16.3 Mutant Protein with Both the N-terminal 35 Residues and C-terminal 9 Residues Truncated Fully Exists As Dimers—In view of the above-described results that the ΔN35 Hsp16.3 mutant proteins exist as both trimers and dimers, whereas the ΔC9 mutant proteins exist predominantly as dimers in addition to minor nonamers, a double mutant (ΔN35ΔC9) with both the N-terminal 35 residues and C-terminal 9 residues truncated was generated. Size-exclusion chromatography and chemical cross-linking results, presented in Fig. 9A and B, clearly demonstrate that the ΔN35ΔC9 mutant protein fully existed as dimers. This dimeric mutant protein, similar to the ΔN35 mutant protein, lacks any chaperone-like activities (data not shown).

It was then asked whether the ΔN35ΔC9 mutant protein could be partially incorporated into the nonamers of the wild-type protein. To address this question, the mixed sample of the ΔN35ΔC9 mutant and the wild-type Hsp16.3 protein were preheated at 65 °C for efficient incorporation (13), cooled to room
temperature, before analyzed at 4 °C on a blue native PAGE (where the proteins are separated primarily based on their differences in both shape and size (41)). The results, shown in Fig. 9C, demonstrate that a small but significant amount of the ΔN35ΔC9 mutant proteins were incorporated into the nonamer under such conditions, as indicated by the decrease in density of the band representing the dimeric mutant protein (compare the band in lane 5 and the bottom band in lane 8) and the corresponding increase in density of the band representing the nonamer form (compare the band in lane 2 and the top band in lane 8). Similar incorporation was also detected for the ΔN35 and ΔC9 mutant proteins (see lanes 2, 3, and 6 for the ΔN35, and lanes 2, 4, and 7 for the ΔC9 in Fig. 9C). It is interesting that the ΔC9 mutant protein was able to form hetero-oligomers besides nonamers with the wild-type subunits (as indicated by the arrows, lane 7, Fig. 9C), suggesting that the C-terminal extension is indeed critical to modulate the oligomerization of Hsp16.3. The higher mobility of the ΔN35ΔC9 protein than the 14-kDa standard protein α-lactalbumin (compare lane 5 with lane 1) may suggest its compact conformation primarily contributed by the α-crystallin domain.
DISCUSSION

Three noteworthy findings in this study of Hsp6.3 are as follows. First, removing the 35 N-terminal residues led to the formation of trimers/dimers lacking chaperone-like activity, suggesting a critical role of this region in the assembly of nonamers from trimers as well as in the exhibition of chaperone-like activity. This finding attributes a role for the N-terminal region of Hsp16.3 somehow similar to that of the \( \text{H}9\text{C}1 \)-crystallin as proposed by de Jong et al. (42). Second, a dimeric mutant protein (\( \text{H}9\text{C}1 \)) of Hsp16.3, with 9 residues from the C-terminal removed, was found to be able to exhibit efficient chaperone-like activity even at room temperature, suggesting that the dissociated small oligomers are the active forms. This finding is in complete accordance with the hypothesis that the nonameric dissociation is a prerequisite for Hsp16.3 to exhibit chaperone-like activity and that the dissociated oligomeric form is active (13–15). Taken together, our accumulative observations, in agreement with reports on Hsp16.5, \( \alpha\text{A} \)- and \( \alpha\text{B} \)-crystallins (19, 43, 44), but unlike those on the \( \text{C. elegans} \) sHSPs (20, 21, 37), \text{Synechocystis} \ sp. PCC 6803 Hsp16.6 (45), and human Hsp20 (38), indicate that the pre-existence of the large oligomer (i.e. nonamer) of Hsp16.3 is not a prerequisite for its chaperone-like activity but probably functions as something of a storage form. Third, the trimers/dimers of the N-terminal deletion mutant (\( \text{N35} \)) can form hetero-nonamers via subunit exchange in a subunit ratio of 2 to 1 between the wild-type and the mutant Hsp16.3 proteins, suggesting that the N-terminal 35 residues in three of the nine subunits in the wild-type Hsp16.3 nonamers are not needed for the assembly of the nonamers from trimers and are thus most likely disordered in structure (analogous to what happens in Hsp16.9).

Several independent observations in this study strongly suggest that the N-terminal region of Hsp16.3 contains the critical site for substrate binding. First, the failure of the \( \text{N35} \) mutant protein to bind bis-ANS indicates that the N-terminal region is not a prerequisite for its chaperone-like activity but probably functions as something of a storage form. Second, the substrate-binding site(s) in Hsp16.3 is overlapped with the bis-ANS binding site(s) (Fig. 5). On the other hand, the substrate-binding site(s) in Hsp16.3 is overlapped with the bis-ANS binding site(s) (Fig. 6). Second, the
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ΔC9 and ΔN35 mutant proteins, although both exist as dissociated oligomers, behave contrary in their respective capacities to exhibit chaperone-like activities and to bind bis-ANS (Figs. 3–5), suggesting that the N-terminal region is critical for Hsp16.3 to form intact substrate-binding site(s). This suggestion is further supported by the behavior of the ΔN35ΔC9 mutant protein. In addition, the Hsp16.3 N-terminal region alone either as a synthesized peptide or in a fusion protein with GST is capable of interacting with denaturing proteins (Fig. 7).

The involvement of the N-terminal region in substrate binding was also implied in other sHSPs. For instance, the N-terminal region of Hsp26 was recently shown to be critical for its interaction with non-native proteins (25, 46). Very similar to the report here, the N-terminal region of Hsp16.5, instead of its C-terminal extension, was found to be necessary for its chaperone-like activity (43). The N-terminal regions in αA-crystallin, αB-crystallin, Hsp18.1, and Hsp16.9 were all proposed to be putative substrate-binding sites as revealed via hydrogen-deuterium exchange, bis-ANS photoincorporation analyses, and crystal structure examination (6, 12, 47, 48). Assuming that such a substrate binding role is true for the N-terminal regions in all sHSPs, then the failure of the N-terminal deletion mutants to exhibit chaperone-like activities for C. elegans Hsp16–2 and the sHSPs from B. japonicum might be explained as reflecting the involvement of this region in substrate binding, rather than the necessity for the formation of large oligomers (26, 37). Likewise, the lack of chaperone-like activities for a few sHSPs found in C. elegans (Hsp12.6, Hsp12.2, and Hsp12.3), naturally existing as either monomers, dimers, or tetramers, is probably caused by the shortening of their N-terminal regions, which would not be able to act as binding sites for denaturing substrate proteins, instead of the failure of the formation of large oligomers (20, 21). Additional supporting evidence also comes from the fact that the N-terminal regions of Hsp16.3, Hsp16.5, Hsp16.9, Hsp18.1, and αA-crystallin are on average more hydrophobic than the rest of each protein (only the hydropathy plot of Hsp16.3 is shown in Fig. 1B; the others are not shown here), being consistent with a previous report on αB-crystallin (48).

The dual role attributed to the N-terminal region of Hsp6.3 in both substrate-binding and the formation of nonamers from trimers would provide an effective mechanism for the small heat shock protein to modulate its chaperone-like activity through oligomeric dissociation/reassociation in responding to environmental conditions as repeatedly implied in our previous investigations (13–15). In addition, it is conceivable that the C-terminal extension, the removal of which leads to the generation of smaller oligomeric forms that exhibit efficient chaperone-like activities even at room temperature, might also play a certain role in modulating the oligomeric state and in turn the chaperone-like activity of the Hsp16.3 protein.

The data showing that at least the N-terminal regions of three subunits in the Hsp16.3 nonamer are not needed for the assembly of nonamers from trimers (Fig. 8) strongly suggests that such N-terminal regions have a disordered structure. In view of this observation on Hsp16.3, in conjunction with what was reported for Hsp16.5 and Hsp16.9 (12, 18, 40), it may be proposed that the presence of disordered N-terminal regions in either part or all of the subunits is a common feature for the large oligomers of sHSPs. Although it is difficult to attribute a role for such disordered N-terminal regions in the assembling of oligomers, there is no difficulty in proposing a role for them in increasing the structural flexibility of the oligomers and broad specificity in binding substrate proteins and/or in releasing the substrate proteins from the small heat-shock proteins for further processing. Such possibilities merit additional investigation.

Although dimeric forms have been reported to be the basic structural and/or functional unit for most sHSPs (12, 18, 19, 43, 45, 49), Mycobacterium tuberculosis Hsp16.3 has been repeatedly reported to exist as nonamers, most likely using trimers as its building blocks, as revealed by studies using dynamic light scattering (27), electron paramagnetic resonance (50), and chemical cross-linking (31). The trimeric form was also observed for the ΔN35 mutant Hsp16.3 protein, and the fact that one third of the subunits in the hetero-oligomers formed between the wild-type and the ΔN35 mutant proteins (with a subunit ratio of 2 to 1) come from the mutant protein (Fig. 8) also strongly implies that the number of subunits in Hsp16.3 oligomers is a multiple of three.

The oligomeric structures of the wild-type and the three mutant Hsp16.3 proteins, as well as the implied roles for the N-terminal region 35 residues and the C-terminal extension 9 residues in the assembly process, are summarized in Fig. 9D, based on observations reported here. The fact that the ΔN35ΔC9 mutant protein (primarily consisting of the α-crystallin domain) fully exists as dimers strongly suggests that structural information for dimer formation, dominant in most other sHSPs, is still present in the wild-type Hsp16.3. It is thus conceivable that, during evolution, the Hsp16.3 trimers were formed from a dimer prototype as a result of the structural information contributed by its N-terminal region and the C-terminal extension. The variation in these two end regions has indeed been suggested to contribute to the diversity of the oligomers (as in shape, size, dynamics) formed for various sHSPs (12, 18, 19, 22, 26, 51).

The subunit ratio of 2:1 in the hetero-nonamers formed between the wild-type protein and the ΔN35 mutant protein strongly suggests the presence of asymmetric trimers in the Hsp16.3 nonamers. Similar asymmetric trimers have been revealed for the E. coli riboflavin synthase based on crystal structure determination, with the asymmetric trimer proposed to be formed from a dimer after binding to a third subunit (52). Based on the behaviors of three mutant Hsp16.3 proteins, we propose that a similar strategy may be used to assemble the asymmetric Hsp16.3 trimers; i.e. a dimer is first formed by the interactions between α-crystallin domains of two subunits, and then the dimer interacts with a third subunit to form the asymmetric trimer, mainly through the interactions contributed by the N-terminal region and the C-terminal extension. Such asymmetric trimers may in turn assemble into symmetric Hsp16.3 nonamers using the N- and C-terminal end regions. The third subunit (the monomeric form) may come from the dissociation of the dimeric form. This hypothesis is somewhat partially supported by the detection of a residual amount of nonamers, co-existing with the dimer and trimer form in an apparently concentration-dependent equilibrium manner, from the wild-type Hsp16.3 proteins produced at an extremely low concentration (about 2.5 μg/ml) by in vitro transcription/translation (see Figs. 1A and 4A from Ref. 31). However, the monomeric form in assembling larger oligomers cannot be detected for Hsp16.3 in the present study, where the protein concentration (about 1 mg/ml) is far higher than the level produced from the in vitro transcription/translation system.

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