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Inter-subunit Cross-linking Suppressed the Dynamic Oligomeric Dissociation of *Mycobacterium tuberculosis* Hsp16.3 and Reduced Its Chaperone Activity

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Abstract—Small heat shock proteins (sHsps) usually exist as dynamic oligomers and oligomeric dissociation was believed to be a prerequisite for their chaperone activities. The truth of this hypothesis was verified in our present study on Hsp16.3, one member of sHsps from *Mycobacterium tuberculosis*, mainly by utilizing chemical cross-linking. Analysis using size exclusion chromatography demonstrated that the heat-induced oligomeric dissociation of Hsp16.3 was severely blocked due to highly efficient inter-subunit cross-linkages generated by chemical cross-linking, as well as its chaperone activity being reduced. Further analysis by non-denaturing pore gradient polyacrylamide gel electrophoresis and fluorescence spectrometry revealed that the dynamic oligomeric dissociation/reassociation process of Hsp16.3 at room temperature was suppressed by inter-subunit cross-linkages, accompanied by significantly decreased exposure of hydrophobic surfaces that are usually hidden in oligomers. These findings supported the hypothesis that substrate-binding sites of sHsps are exposed presumably by dissociation of larger oligomers into smaller active oligomers, and therefore such a dissociation process could be adjusted to modulate chaperone activities.

Key words: *Mycobacterium tuberculosis*, Hsp16.3, dissociation, chaperone activity

Small heat shock proteins (sHsps), one subclass of molecular chaperones, are found in varying concentrations in all organisms [1]. In response to elevated temperatures or other stress conditions, the expression of sHsps is strongly increased [2]. In addition, over expressed recombinant sHsps are found to increase the thermal tolerance of *E. coli* cells [1, 3]. *In vitro* studies indicated that sHsps could tightly bind aggregation-prone denaturing proteins and form stable complexes [4-6], which act as a reservoir of denaturing intermediates for reactivation with the help of other chaperone systems such as GroEL and Hsp70 [5, 7, 8].

One common feature of sHsps is that they exist as dynamic oligomers composed of 9-40 subunits, which are found to undergo subunit exchange under physiological conditions [9-14]. Ample evidence has suggested that sHsps such as Hsp26, Hsp16.3, α-crystallin, and Hsp16.9 expose their hydrophobic substrate-binding sites or function as molecular chaperones presumably by dissociation of larger oligomers into smaller subunits [12, 13, 15-18]. Especially in our recent study [19], Hsp16.3 was found to be able to modulate its chaperone activity by adjusting the rate of oligomeric dissociation.

The hypothesis that the oligomeric dissociation is a prerequisite for sHsps to exhibit chaperone activities is based on some similar observations, i.e., a correlation between the heat-induced oligomeric dissociation and enhanced chaperone activities. To further provide strict evidence for this hypothesis, an alternative approach was taken in this study using small heat shock protein Hsp16.3 from *Mycobacterium tuberculosis* as a model system: block the oligomeric dissociation process by inter-subunit cross-linking and in turn suppress its chaperone activity. We obtained highly efficient inter-subunit cross-linked Hsp16.3 oligomers and showed that such cross-linked species cannot efficiently function as molecular chaperones, most likely due to its blocked oligomeric dissociation. Therefore, our results strongly support the hypothesis that the oligomeric dissociation process of sHsps is a prerequisite for their chaperone activities to appear.

Abbreviations: sHsps) small heat shock proteins; Hsp16.3) 16.3-kD small heat shock protein from *Mycobacterium tuberculosis*; SEC) size exclusion chromatography; DTSSP) 3,3′-dithiobis(sulfosuccinimidyl)propionate; ANS) 8-anilino-1-naphthalene sulfonate.

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MATERIALS AND METHODS

Materials. 3,3′-Dithiobis(sulfosuccinimidyl)propionate (DTSSP) was purchased from Pierce Biotechnology (USA). Catalase, lysozyme, BSA, and 8-anilino-1-naphthalene sulfonate (ANS) were all obtained from Sigma (USA). Hemoglobin O from *Mycobacterium tuberculosis* was purified in our lab. All other chemical reagents were of analytical purity.

Protein purification. The gene for Hsp16.3 of *M. tuberculosis* was cloned into the pET-9d vector for protein expression as previously described [20]. The recombinant Hsp16.3 protein was over-expressed in BL21(DE3) *E. coli* cells transformed with the plasmid and purified according to methods as previously described [13]. The proteins were dialyzed in distilled and deionized water, lyophilized, and stored at −20°C before use. Protein concentrations were determined using the Bio-Rad Protein Assay (USA).

Preparation of cross-linked Hsp16.3 proteins. The cross-linked Hsp16.3 proteins were prepared using DTSSP, a water-soluble thiol-cleavable and amine-reactive cross-linker, according to the supplier's instructions. Briefly, the reaction was conducted in 50 mM sodium phosphate buffer, pH 7.0, at room temperature for 1 h and stopped by quenching with 1 M Tris-HCl, pH 7.0, for 10 min. The residual DTSSP and Tris-HCl were removed by centrifugation (at 14,000*g* for 30 min) using a Microcon YM-10 tube (Millipore, USA), with the concentrated proteins being washed twice with 50 mM sodium phosphate buffer.

Circular dichroism (CD) spectroscopy. CD spectra were recorded on a J-715-150L spectropolarimeter (Jasco, Japan) equipped with a water bath. Protein samples (300 μl, 0.2 mg/ml protein for far-UV CD or 2 mg/ml proteins for near-UV CD) in 2-mm quartz cuvettes were equilibrated at 25°C for 10 min before scanning. The spectra were recorded with a 1-nm bandwidth and a 0.5-nm step size at a rate of 1 nm/sec and averaged over 5 runs.

Temperature-controlled size exclusion chromatography (SEC). Temperature-controlled SEC was performed on a AKTA FPLC system using a XK 16/70 column self-packed with Superdex 200 prep grade medium (all from Amersham Pharmacia Biotech, USA). The column efficiency was 11,567 plates/meter and the asymmetry was 1.03 when sampled on acetone. The temperature of chromatography was controlled by connecting the thermostat jacket of the column to a water-bath (Multitemp III, Amersham Pharmacia Biotech) and preheating the column at the indicated temperatures for 1 h. For each analysis, a 100-μl protein sample (of 5 mg/ml) was loaded (centrifuged before loading). Proteins were eluted with 50 mM sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl at a flow-rate of 1 ml/min.

Chaperone activity assay. Chaperone activities of the untreated and DTSSP cross-linked Hsp16.3 protein were assayed by measuring their capacity to suppress the thermally induced aggregation of catalase. The aggregation was monitored at 360 nm on a UV-8500 spectrophotometer (Shanghai Techcomp, China). In order to obtain an optimal absorbance at 360 nm (between 1 and 2) to follow the protein aggregation process at the indicated temperatures, the final concentrations of catalase (in 50 mM sodium phosphate buffer, pH 7.0) applied at 45, 55, and 65°C were 80, 40, and 30 μM, respectively. Correspondingly, the respective concentrations of Hsp16.3 monomers were 20, 5, and 2.5 μM. The chaperone activities for each measurement were calculated as $(A_0 - A)/A_0$, where A_0 and A, respectively, represent the values of absorbance when the thermal aggregation curves reach their plateau stages (after about 2 h of heating) in the absence and presence of Hsp16.3 proteins. The relative chaperone activities of DTSSP-cross-linked Hsp16.3 proteins at each indicated temperature were calculated by taking those of the uncross-linked Hsp16.3 proteins as 100%.

Pore-gradient polyacrylamide gel electrophoresis (PAGE). Non-denaturing pore-gradient PAGE with a linear gradient concentration from 4-30% was prepared in a 125 × 100 × 1 mm mold as described before [13]. The electrophoresis was performed at constant electric current of 2 mA. The samples of proteins were separated on a single gel, for 14 or 18 h, respectively.

ANS fluorescence assay. The fluorescence of ANS after binding to Hsp16.3 was measured on a Hitachi F-2500 fluorescence spectrophotometer (Japan). Hsp16.3 protein (0.08 mg/ml) in 50 mM sodium phosphate buffer, pH 7.2, was incubated with 20 μM ANS at 25°C for 10 min before performing fluorescence recording. The ANS emission was scanned between 400-600 nm while excited at 390 nm.

RESULTS

Highly efficient inter-subunit cross-linking blocked the heat-induced oligomeric dissociation of Hsp16.3. Heat-induced oligomeric dissociation was found to enhance the chaperone activities of sHsps [13, 15]. Our recent study further revealed that Hsp16.3 could modulate its chaperone activities by adjusting the rate of oligomeric dissociation [19]. To further demonstrate the necessity of such oligomeric dissociation for the increment of chaperone activities, the opposite approach was taken here: inter-subunit cross-linkages were introduced to suppress the dissociation process, using amine-reacting agent DTSSP [13]. In agreement with our expectation, size exclusion chromatography analysis (Fig. 1) clearly demonstrated that temperature-dependent oligomeric dissociation of Hsp16.3 was blocked after inter-subunit cross-linking. Upon increasing the temperature from 25 to 65°C, the uncross-linked Hsp16.3 has dissociated completely from nonamers into smaller oligomers (possibly

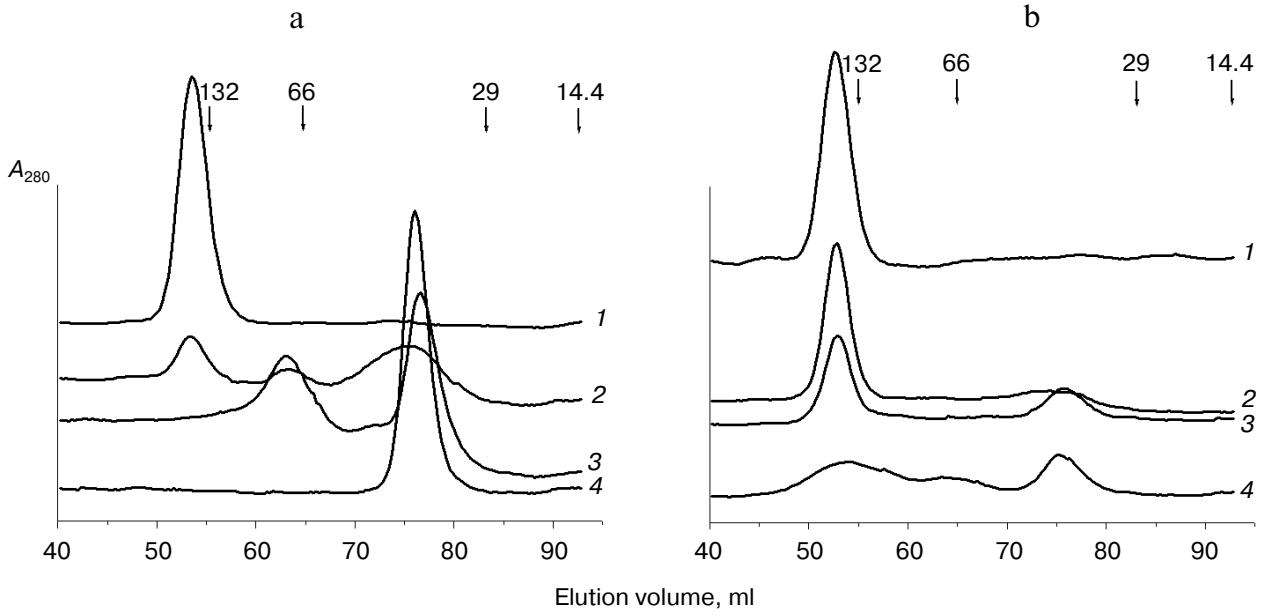

Fig. 1. Inter-subunit cross-linking blocked the heat-induced oligomeric dissociation of Hsp16.3. The elution curves of the size exclusion chromatography for the uncross-linked (a) and cross-linked proteins (b), respectively, performed at the indicated temperatures: 25 (*1*), 45 (*2*), 55 (*3*), and 65°C (*4*). The elution positions of the molecular mass standards (BSA dimer of 132 kD, BSA monomer of 66 kD; hemoglobin O from *M. tuberculosis* of 29 kD; and the hen lysozyme of 14.4 kD) are indicated by the downward arrows.

being trimer, as revealed in our previous study [21]) with certain oligomers as intermediates (Fig. 1a). In contrast, some of the cross-linked Hsp16.3 still exist as nonamers even if the temperature is increased up to 65°C (Fig. 1b).

The cross-linking efficiency was higher than 50% as estimated by examining the density reduction of the monomeric forms and the appearance of the various oligomeric forms in the cross-linked samples using SDS-PAGE (Fig. 2a, lanes *2* and *3*). The size exclusion chromatography analysis meanwhile revealed no change in the oligomeric size after such cross-linking (as shown by the top spectra in Fig. 1, a and b). Similarly, examination of the protein samples using CD spectroscopy revealed no detectable changes at the level of secondary and tertiary structures (Fig. 2, b and c).

Inter-subunit cross-linking lowers the chaperone activity of Hsp16.3 at high temperature. The observation of blocked oligomeric dissociation for Hsp16.3 by inter-subunit cross-linking (as shown in Fig. 1) raises an interesting question: whether the chaperone activity of cross-linked Hsp16.3 will be hereby suppressed simultaneously? The results presented in Fig. 3 demonstrated that the chaperone activities of Hsp16.3 at high temperatures were reduced mostly due to inter-subunit cross-linkages, as reflected by the following facts. 1) No significant difference in the chaperone activity between the cross-linked and uncross-linked Hsp16.3 proteins was observed when measured at 45°C. 2) At 55°C and especially at 65°C, the level of chaperone activity for the cross-linked Hsp16.3 is obviously lower than that for the uncross-linked protein. Considering that no significant change was observed for the cross-linked Hsp16.3 on the level of native secondary, tertiary, and quaternary structures, it is reasonable that the reduction of chaperone activities could be correlated with the suppressed oligomeric dissociation as a result of inter-subunit cross-linkages.

It is noted that in our previous study [13], Hsp16.3 exhibited enhanced chaperone activity after cross-linking or after reduction of cross-linkages, which was suggested to disturb the structure of Hsp16.3 and in turn enhance the chaperone activity. Such paradoxical phenomena, seeming to be opposite to the results here (Fig. 3), could be explained as following: the effectiveness of cross-linking in that report [13] was too low to block the oligomeric dissociation process such that the positive effect (disturbing the structure of Hsp16.3) was stronger than the negative effect (blocking the oligomeric dissociation of Hsp16.3), subsequently increasing the chaperone activity of Hsp16.3. However, in the present study, we obtained highly efficient cross-linked Hsp16.3 (Fig. 2a), of which the oligomeric dissociation process was effectively blocked (Fig. 1). Therefore, the present study, following our expectation, demonstrated the prerequisite of oligomeric dissociation of Hsp16.3 for its chaperone activity to appear.

Inter-subunit cross-linking suppressed the exposure of hydrophobic surfaces of Hsp16.3 by blocking the dynamic oligomeric dissociation/reassociation process. Our previous studies [13, 19] have demonstrated that Hsp16.3

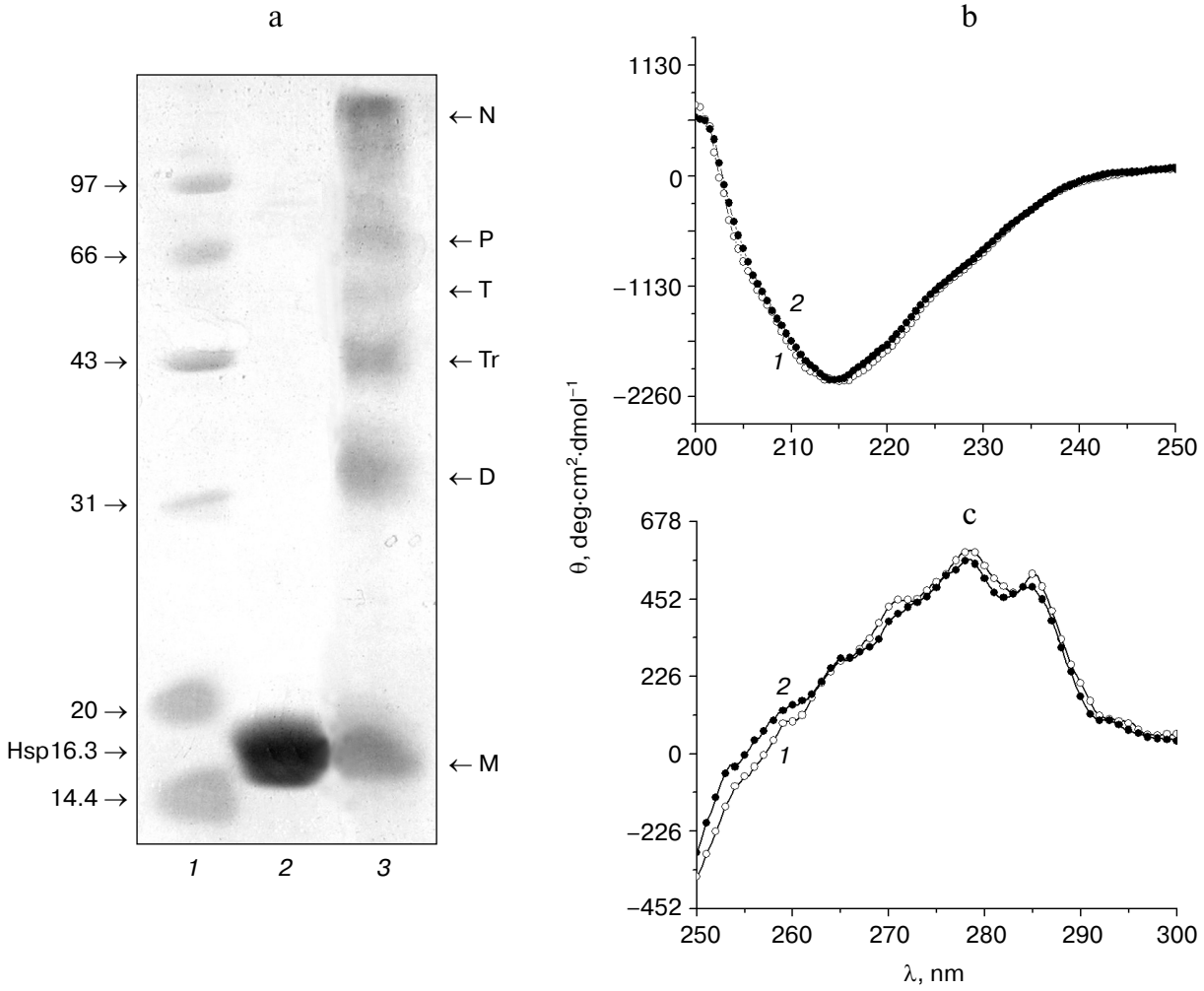

Fig. 2. Effect of inter-subunit cross-linking on Hsp16.3 structure. The two proteins (uncross-linked and cross-linked Hsp16.3) were applied to SDS-PAGE visualized by Coomassie brilliant blue staining, far-UV CD and near-UV CD spectroscopy analysis (a, b, and c, respectively). a) Lanes: *1*) protein standards (on the left, molecular masses in kD); *2*) uncross-linked Hsp16.3; *3*) cross-linked Hsp16.3. The positions of cross-linked oligomers—dimer (D), trimer (Tr), tetramer (T), pentamer (P), and nonamer (N)—are indicated on the right (M is monomer). Curves *1* and *2* in panels (b) and (c) represent the uncross-linked and cross-linked Hsp16.3 proteins, respectively.

always appears as a smear when separated on a non-denaturing pore-gradient PAGE. In such an electrophoresis system, multimeric proteins will eventually reach their respective "pore limits" determined by their molecular size, thus forming sharp bands [22]. However, for oligomeric proteins like Hsp16.3 that exists as a dynamic dissociation/reassociation oligomers [13, 19], it will continuously dissociate into smaller oligomers after reaching its "pore limits", thus forming a gradient smear rather than a sharp band. Such gel electrophoresis system was applied to further analyze the differences of the various Hsp16.3 proteins in terms of their capability of dynamic dissociation/reassociation (see also [19]).

Results presented in Fig. 4a demonstrated that the dynamic dissociation/reassociation of cross-linked Hsp16.3 was significantly weaker than that of uncross-linked proteins as reflected by the following observations. 1) The cross-linked Hsp16.3 was separated mainly in the form of nonamer (lanes *3* and *6*) with slight smears. 2) The uncross-linked Hsp16.3 was separated to form a gradient smear extending from nonamer to smaller oligomers (lanes *2* and *5*), indicating that it undergoes rapid dynamic oligomeric dissociation/reassociation as revealed in our previous study [13, 19]. 3) When the electrophoresis time is prolonged (from 14 to 18 h), some of uncross-linked Hsp16.3 appears to dissociate into smaller oligomers (lane *2*); however, the cross-linked Hsp16.3 nearly retains its nonameric size (lane *3*).

Ample evidence has been provided to suggest that the substrate-binding sites in sHsps are hydrophobic patches [16, 18, 23], which could be detected using hydrophobic probes such as 1,1′-bi(4-anilino)naphthalene-5,5′-disul-

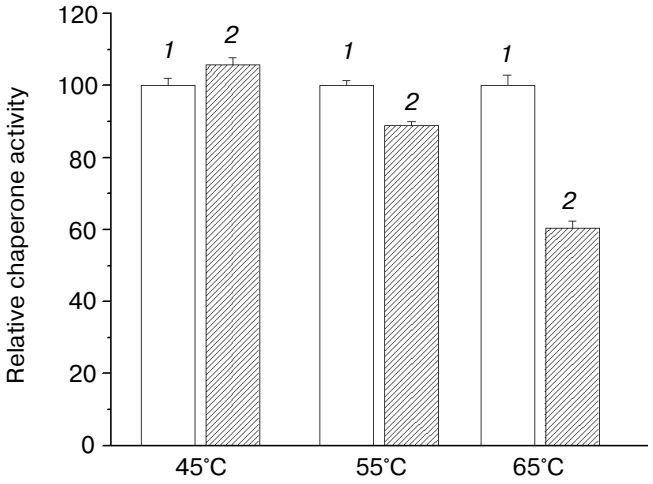

Fig. 3. Inter-subunit cross-linking lowers the chaperone activity of Hsp16.3 at high temperatures. The relative chaperone activities of the uncross-linked protein (*1*) and cross-linked Hsp16.3 (*2*) in suppressing the thermal aggregation of catalase at various temperatures. Chaperone activity of the uncross-linked protein is taken as 100%, with the calculating details described in the "Materials and Methods".

fonic acid (bis-ANS) and ANS: higher fluorescence intensity, more exposure of hydrophobic surfaces in proteins. Given that the hydrophobic substrate-binding sites for sHsps are exposed presumably by the oligomeric dissociation, the exposure of hydrophobic surfaces for cross-linked Hsp16.3 is expected to be decreased due to its blocked dynamic oligomeric dissociation as shown in Fig. 4a. In agreement with our expectation, the result presented in Fig. 4b demonstrated that the cross-linked Hsp16.3 exposed significantly lower hydrophobic surfaces: the fluorescence intensity of ANS after binding cross-linked Hsp16.3 is much lower than that after binding the uncross-linked protein. Such reduced exposure of hydrophobic surfaces by blocked dynamic oligomeric dissociation further supports the idea that the oligomeric dissociation of Hsp16.3 is a prerequisite for its substrate-binding sites to expose and chaperone activities to appear.

DISCUSSION

Our studies reported here, using chemical cross-linking that is a different approach from those previously used, strongly demonstrated that the oligomeric dissociation process is a key feature that determines the level of chaperone activity for Hsp16.3. We first obtained highly efficient inter-subunit cross-linked Hsp16.3 (Fig. 2a) and ruled out the possibility that cross-linking itself will disturb the native structure of Hsp16.3 (Fig. 2, b and c). Second, we found that the heat-induced oligomeric dissociation and chaperone activity of Hsp16.3 were both suppressed by inter-subunit cross-linking (Figs. 1 and 3). Further studies indicated that the dynamic oligomeric dissociation/reassociation process at room temperature

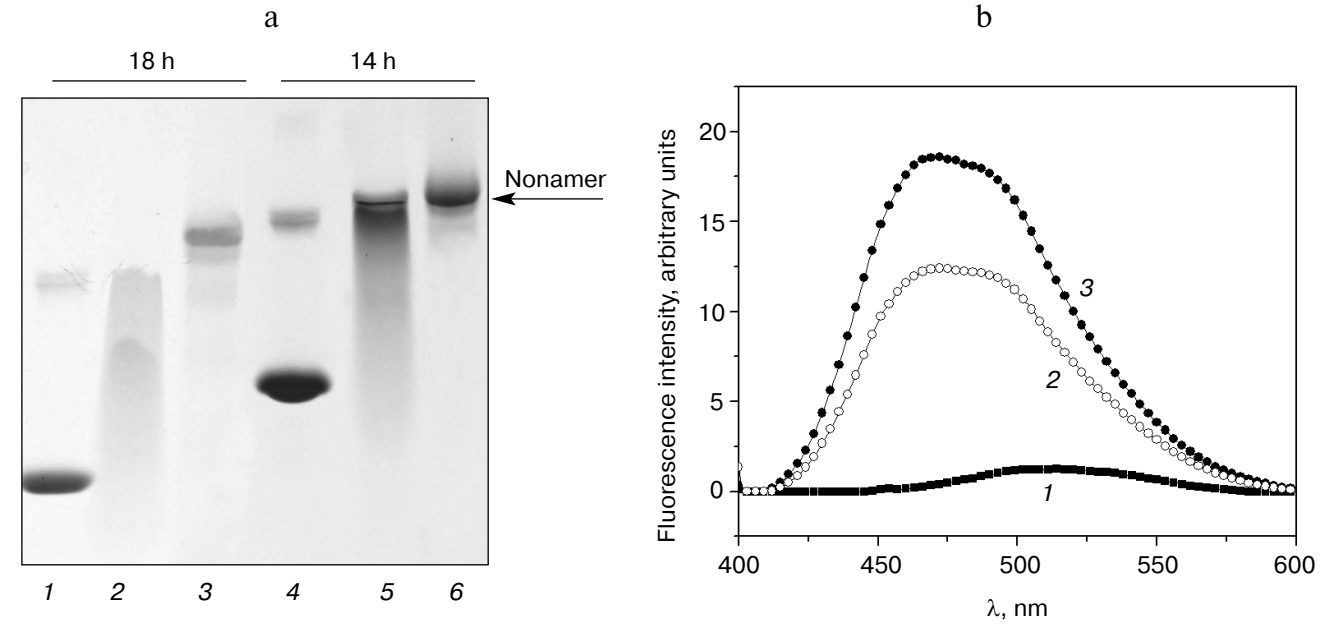

Fig. 4. The exposure of hydrophobic surfaces of Hsp16.3 and the dynamic dissociation/reassociation process were both suppressed by inter-subunit cross-linking. a) The uncross-linked and cross-linked Hsp16.3 proteins were analyzed by non-denaturing pore gradient PAGE at room temperature, with different electrophoresis time (14 and 18 h, respectively) and visualized by Coomassie brilliant blue staining. Lanes: *1*, *4*) BSA; *2*, *5*) uncross-linked Hsp16.3; *3*, *6*) cross-linked Hsp16.3. b) Fluorescence spectra of ANS (20 μM) at 25°C in the absence of proteins (*1*), or after binding uncross-linked (*2*) or cross-linked (*3*) Hsp16.3 proteins (0.08 mg/ml).

was also blocked by inter-subunit cross-linking, accompanied by reduced exposure of hydrophobic surfaces. Collectively, these findings strongly support the hypothesis that the oligomeric dissociation process is a prerequisite for sHsps to expose substrate-binding sites and function as molecular chaperones.

The use of oligomeric dissociation to expose substrate-binding sites by sHsps might have the following advantages. 1) Given that the exposure of buried hydrophobic regions is believed to be the cause for the aggregation of proteins [24], the hydrophobic substrate-binding sites of sHsps are usually hidden in large oligomers [18], thus preventing sHsps themselves from aggregation. 2) sHsps undergo reversible and rapid oligomeric dissociation and re-association, and therefore the exposure of hydrophobic patches are transient rather than permanent, as revealed in other studies [12, 25]. In addition, the high conformational flexibility of sHsps also assists themselves to regulate the exposure of hydrophobic surfaces freely and reduce some non-native interaction as analyzed in our previous study [26]. As a consequence, the aggregation for sHsps (as well as for other molecular chaperones such as Hsp60, Hsp70) themselves never occurs under both non-denaturing and denaturing conditions, although hydrophobic surfaces are thought to be intrinsic parts of molecular chaperones. 3) Given that the inter-subunit contacts in sHsps oligomers are mainly contributed by hydrophobic interaction, ionic interactions, and inter-subunit hydrogen bonds [17, 18], the change in environmental conditions would affect such kinds of noncovalent interactions and in turn regulate the oligomeric dissociation process. In other words, sHsps are able to adjust the oligomeric dissociation process and subsequently modulate chaperone activities in response to the changes of environmental conditions as demonstrated for Hsp26, Hsp16.3, and α-crystallin [13, 15, 16, 19]. Such regulatory mechanism could assist sHsps to instantly function as molecular chaperones and provide immediate protection for denaturing proteins while stress conditions appear [15, 18, 19].

Our results reported here might also be applied to explain the similar observations for human lens α-crystallin, which was found to form covalent bonds during aging or cataractogenesis of the human lens and exhibit significantly diminished chaperone activity [27-29]. Although the reduction of chaperone activity for α-crystallin was attributed to the conformational change generated by covalent bonds in those studies, it is more conceivable that the suppressed oligomeric dissociation of α-crystallin by inter-subunit covalent bonds resulting from post-translation modifications, being similar with our chemical cross-linking results, reduced its chaperone activities.

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