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Identification of bis-ANS binding sites in *Mycobacterium tuberculosis* small heat shock protein Hsp16.3: Evidences for a two-step substrate-binding mechanism ☆

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Abstract

Small heat shock proteins (sHSPs), as one important subclass of molecular chaperones, are able to specifically bind to denatured substrate proteins rather than to native proteins, of which their substrate-binding sites are far from clear. Our previous study showed an overlapping nature of the sites for both hydrophobic probe 1,1′-Bi(4-anilino)naphthalene-5,5′-disulfonic acid (bis-ANS) binding and substrate binding in *Mycobacterium tuberculosis* Hsp16.3 [X. Fu, H. Zhang, X. Zhang, Y. Cao, W. Jiao, C. Liu, Y. Song, A. Abulimiti, Z. Chang, A dual role for the N-terminal region of *M. tuberculosis* Hsp16.3 in self-oligomerization and binding denaturing substrate proteins, J. Biol. Chem. 280 (2005) 6337–6348]. In this work, two bis-ANS binding sites in Hsp16.3 were identified by a combined use of reverse phase HPLC, mass spectroscopy and N-terminal protein sequencing. One site is in the N-terminal region and the other one in the N-terminus of α-crystallin domain, both of which are similar to those identified so far in sHSPs. However, accumulating data suggest that these two sites differentially function in binding substrate proteins. With regard to this difference, we proposed a two-step mechanism by which Hsp16.3 binds substrate proteins, i.e., substrate proteins are recognized and initially captured by the N-terminal region that is exposed in the dissociated Hsp16.3 oligomers, and then the captured substrate proteins are further stabilized in the complex by the subsequent binding of the N-terminus of α-crystallin domain.

Keywords: Chaperone; Small heat shock protein; Hsp16.3; N-terminal region; bis-ANS

Small heat shock proteins (sHsps), as one sub-class of molecular chaperones, have been found in nearly all organisms [1,2] with their expression inducible in response to elevated temperatures or other stress conditions [3]. In primary structure, the sHsps are characterized by the presence of a conserved α-crystallin domain that is preceded by an N-terminal region of variable length and sequence and followed by a short C-terminal extension [2,4,5]. *In vitro* studies demonstrated that sHsps are able to exhibit chaperone-like activities, preventing aggregation of denatured proteins by forming tightly bound complexes with them.

A model involving the oligomeric dissociation and re-association of sHSPs has been proposed to explain the mechanism underlying chaperone-like activities [6–10]. On the other hand, special primary sequences of sHSPs involved in binding substrate proteins also have been identified in some members [6,11,12]. However, it is far from clear whether substrate-binding sites play different roles in binding substrate proteins and how they function in the context of oligomeric dissociation of sHSPs. Hsp16.3, the small heat shock protein from *Mycobacterium tuberculosis* [13],

☆ *Abbreviations:* bis-ANS, 1,1′-Bi(4-anilino)naphthalene-5,5′-disulfonic acid.

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was studied here to clarify this question. Our previous studies revealed that Hsp16.3 exhibits temperature-dependent chaperone-like activities, with the oligomeric dissociation as a prerequisite [8,10,14], and that its N-terminal region plays a dual role in both self-oligomerization and substrate proteins binding [9]. This protein was recently found to exist as dodecamers by using dimers as building blocks [15].

This study here represents our continuous effect on Hsp16.3. We determined substrate-binding sites of Hsp16.3 by using 1,1′-Bi(4-anilino)naphthalene-5,5′-disulfonic acid (bis-ANS) given that this hydrophobic probe is able to bind to substrate binding sites in sHSPs including Hsp16.3 [6,9,12,16]. Two bis-ANS bindings sites in Hsp16.3 were identified. However, accumulating data indicate that these two substrate-binding sites differentially function in binding substrate proteins. Based on this observation and other data [8–10,13–15], we propose that Hsp16.3 might recognize and initially capture substrate proteins by using its first binding site after its oligomeric dissociation, and then further stabilize the captured substrate proteins through the second binding site. Such a quaternary structural change coupling with a two-step substrate-binding model might give us insights into understanding the mechanism of sHSPs underlying chaperone-like activity.

Materials and methods

Materials. Bis-ANS and trypsin were obtained from Sigma. The Hsp16.3 was purified as previously described [13].

Photo-incorporation of bis-ANS into Hsp16.3 proteins. Photo-incorporating bis-ANS into Hsp16.3 proteins was carried out according to methods previously described [12,17]. Briefly, the Hsp16.3 protein (0.4 mg/ml) was incubated with bis-ANS (100 μM) at 35 °C or at 65 °C for 10 min before irradiated in a UVC 500 cross-linker (Amersham Pharmacia Biotech, with the power being 120,000 μJ/cm^2) for 20 min with continued incubation at 35 °C or at 65 °C.

Bis-ANS fluorescence intensity assay. The fluorescence intensity of bis-ANS was measured by scanning between 450 and 560 nm after being excited at 390 nm on a Hitachi F-4500 fluorescence spectrophotometer.

Identification of bis-ANS labeled peptide fragments in Hsp16.3. The Hsp16.3 wild type protein (0.4 mg/ml) was labeled with 100 μM bis-ANS at 65 °C as described above. The residual bis-ANS was removed by Microcon YM-3 tube (Millipore) (centrifuged at 14,000*g* for 30 min), with the proteins washed three times using 20 mM Tris–HCl (pH 8.0). The bis-ANS labeled Hsp16.3 protein (approximately 170 μg) was digested at 37 °C for 2 h, with the ratio of trypsin to Hsp16.3 being at 1:20. The peptide fragments were separated by reverse phase HPLC (Chemstation 1100 series, Agilent) on a C18 column equilibrated with 0.065% trifluoroacetic acid (in H_2O). In order to achieve better resolution, the elution (at a rate of 1 ml/min) was performed as follows: the concentration of acetonitrile was first increased from 0% to 10% over 6 min, then maintained at 10% for 5 min, subsequently increased from 10% to 50% over 120 min, and finally increased from 50% to 85% over 10 min. The peak fractions were collected manually. The bis-ANS labeled peptide fragments were identified by using the fluorescence assay described above. The molecular mass of peptides displaying significant fluorescence was determined using an API3000 Electronic Spray Ionization Mass Spectrometry (ESI-MS) System (Applied Biosystems), and the N-terminal amino acid sequences (for 3 residues) were determined using an API491 protein sequencer (Applied Biosystems).

Size exclusion chromatography. Size exclusion chromatography was performed on a ÄKTA FPLC system using 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 with 50 mM phosphate sodium buffer (containing 0.15 M NaCl, pH 7.0) at a flow-rate of 0.5 ml/min.

Results

Identification of bis-ANS binding sites in Hsp16.3

Bis-ANS, a widely used hydrophobic probe, has been demonstrated to be able to bind to amino acids in sHSPs that are involved in binding denatured proteins [6,12,16]. Our previous studies also showed an overlapping nature of the sites in Hsp16.3 for both bis-ANS binding and substrate binding [9]. Here, the bis-ANS binding sites in Hsp16.3 were identified via a combined use of trypsin digestion on Hsp16.3 that was photo-incorporated with bis-ANS, subsequent peptides separation by reverse phase HPLC, as well as of peptides determination by mass spectroscopy and N-terminal protein sequencing.

Among the five peptide fragments that exhibited significant fluorescence (Fig. 1A), N-terminal sequences and masses were obtained for three of them (the other two failed to give signals when applied to mass spectrometry analysis, likely due to the too low concentration). The ESI-MS analysis revealed the molecular masses of the three peptide fragments a, c, and e to be 5203, 4209, and 2952 Da, respectively (Fig. 1B). The Edman degradation analysis revealed the N-terminal three residues for peptides a, c, and e to be AEL, ATT, and SLF, respectively (the detailed data not shown). The three peptides were definitely matched along the amino acid sequence of Hsp16.3 (as indicated in Fig. 1C) after a combined analysis on the N-terminal sequences, the molecular masses (with the molecular mass of one labeled bis-ANS, 679.2 Da, being subtracted) and the specific trypsin cleavage sites present in the protein. Two peptides are overlapped within the N-terminal region (amino acids 2–32) and the third peptide (amino acids 55–84) in the N-terminus of α-crystallin domain (Fig. 1C).

Photo-incorporation of bis-ANS into Hsp16.3 does not change its oligomeric size

We previously suggested that the structure of sHSPs, particularly of their substrate-binding sites, is highly flexible, and such a property is necessary for their chaperone-like activities [9,18]. Here we tested this idea by examining the effect of photo-incorporation of bis-ANS on the oligomeric size of Hsp16.3. The size exclusion chromatography analysis as presented in Fig. 2 (comparing curve 1 with 2) clearly demonstrated that the photo-incorporation of bis-ANS into Hsp16.3 at 35 °C does not affect the oligomeric size of this protein, thus showing that the oligomeric structure of Hsp16.3 is highly flexible to the occupation of bis-ANS. Of particularly interesting, dissociated Hsp16.3 oligomers after the photo-incorporation of bis-ANS performed at 65 °C are still able to re-assemble into

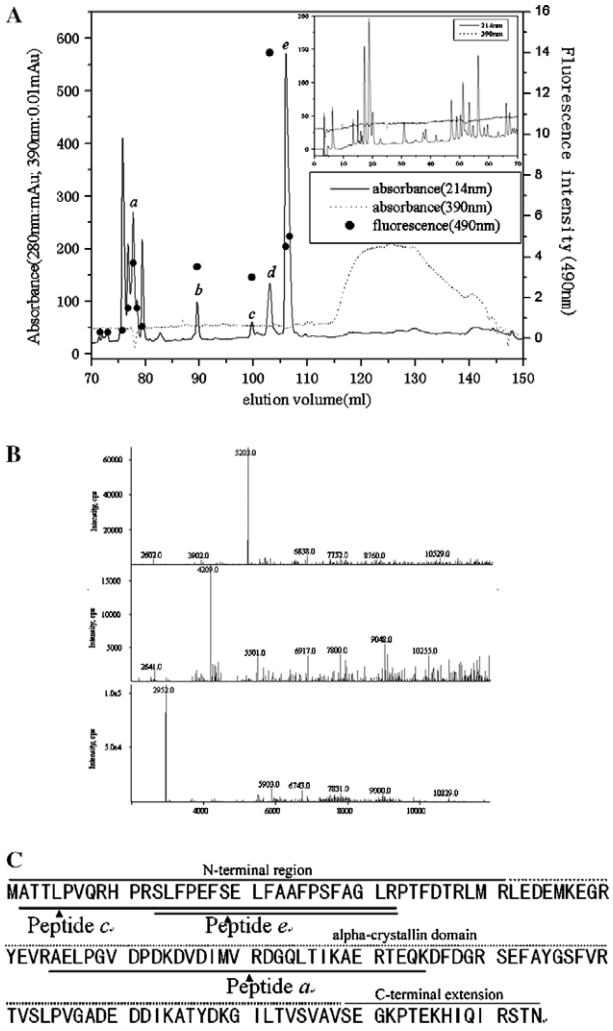

Fig. 1. Separation and identification of bis-ANS binding peptides. (A) Reverse phase HPLC elution curves for tryptic digests of bis-ANS labeled Hsp16.3 wild type as described in Materials and methods. The light absorbance at 214 nm (for peptides) and 390 nm (for bis-ANS) against elution volume (70–150 ml) was shown, as well as the fluorescence (390 excitation/490 emission) of isolated peaks. Peaks a, b, c, d, and e with significant higher fluorescence were designated. The inset was shown the absorbance curves with the elution volume ranging from 0 to 70 ml, of which there was no significant fluorescence in the isolated peaks. (B) The mass spectroscopy analysis for the peptide a, c, and e, respectively. (C) The sequences of peptide a, c, and e in Hsp16.3 deduced from the results shown in (A) and (B). The N-terminal region, α-crystallin and C-terminal extension are indicated as described in Ref [9].

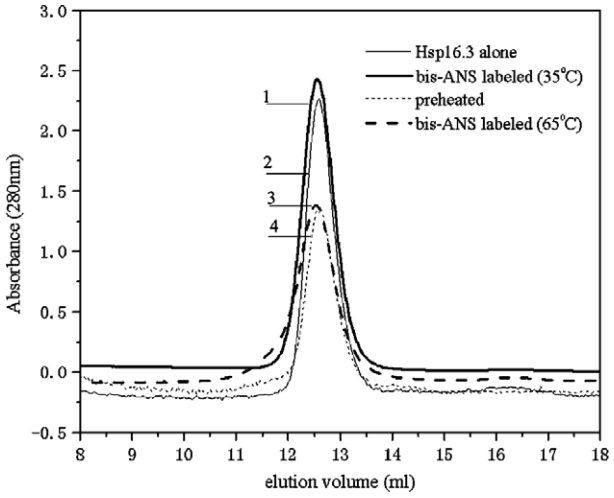

Fig. 2. Photo-incorporation of bis-ANS does not change the oligomeric size of Hsp16.3. The size exclusion chromatography elution curves (performed at room temperature) of unlabeled or bis-ANS labeled Hsp16.3 proteins. Hsp16.3 (0.4 mg/ml) was incubated with or without bis-ANS (100 μM) at indicated temperature for 10 min, irradiated at the same temperature for 20 min, and then cooled down at room temperature for 10 min before chromatography analysis.

the native oligomer (Fig. 2, comparing curve 3 with 4), thus strengthening our previous view that Hsp16.3 protein possesses the high ability of spontaneously refolding/re-assembly originated from its high structural flexibility [9,18]. Assuming that bis-ANS is an analog of small substrate proteins, it is reasonably extrapolated that the whole oligomeric structure is flexible to accommodate substrate proteins.

Discussion

In this work, bis-ANS binding sites in Hsp16.3 were identified in the N-terminal region and the N-terminus of α-crystallin domain. The data are in accordance with our previous observation that both sites were necessary for Hsp16.3 to exhibit chaperone-like activities [9]. Particularly important, the N-terminal region was repeatedly identified as bis-ANS binding sites (Fig. 1C), thus nicely supporting the previous conclusion that this region plays a critical role in binding denatured substrate proteins for Hsp16.3 [9].

Although the N-terminal region of Hsp16.3 and its N-terminus of α-crystallin domain are found here as potential substrate-binding sites, our series studies [9,19,20] showed that these two sites might play different roles in binding denatured substrate proteins. First, the N-terminal region alone is indeed capable of recognizing and interacting with denatured substrate proteins [9]. In contrast, the N-terminus of α-crystallin domain fails to interact with substrate proteins at all, indicating that its binding to substrate proteins requires the assistance from other structural components. Second, structural studies showed that the N-terminal region of Hsp16.3 most likely exists as disordered structures [9] while the N-terminus of α-crystallin domain is involved in the formation of β-sheets [19,20]. Third, the hydrophobicity of the N-terminal region is much higher than that of the N-terminus of α-crystallin domain [9], suggesting that the interaction of the former one to substrate proteins is stronger than that of the latter one given that hydrophobic interaction plays dominant roles between sHSPs and substrate proteins [16,17,21,22].

In light of such differences between these two substrate-binding sites, together the previously proposed mechanism involving the oligomeric dissociation of Hsp16.3 [8,14], we propose a two-step substrate binding hypothesis, in which substrate proteins are recognized and initially captured by the first binding site, i.e., the N-terminal region of Hsp16.3

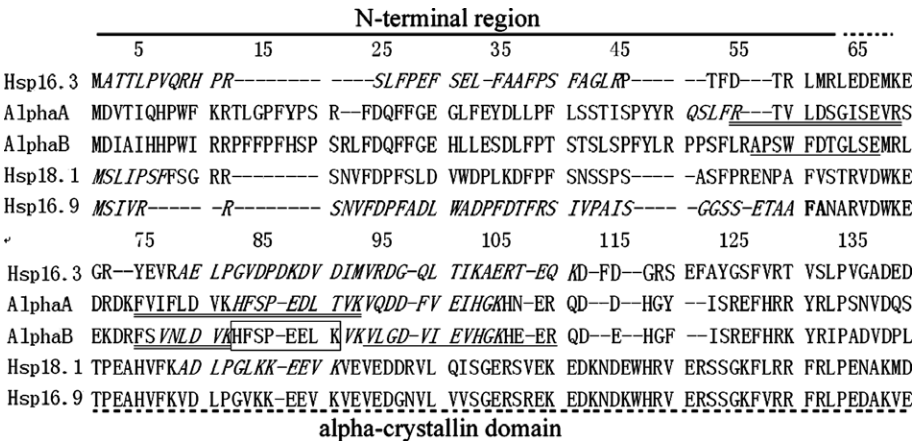

Fig. 3. Substrate-binding sites comparison between Hsp16.3 and other sHSPs. The hydrophobic probe or substrate-bindings sites are indicated in the full sequence alignment of these sHSPs, with the C-terminus of α-crystallin domain, and the C-terminal extension being omitted for simplicity. The bis-ANS binding sequence in Hsp16.3 identified in this work, as well as that in αA-crystallin and αB-crystallin [12] and pea Hsp18.1 [17] are shown in italic. The 1, 5-AZNS binding sequence in αB-crystallin [12] is boxed. The alcohol dehydrogenase binding sites in αB-crystallin are marked with an underline [11]. The melittin-binding sites in αA-crystallin and αB-crystallin [27] are marked with double underline. The N-terminal arm in Hsp16.9 (shown in italic) was suggested as a putative substrate-binding site [24].

that is fully exposed when the dodecamer dissociates into small oligomers [9,10,15], and then the captured substrate proteins are further bound in the complex by the second binding site, i.e., the N-terminus of α-crystallin domain. In this hypothesis, the first step binding should play predominant roles in binding denatured substrate proteins due to the reasons presented above.

Some illusive phenomena can be partially clarified by using this hypothesis. For an instance, although the first binding site of Hsp16.3 alone was able to recognize and bind denatured proteins, it failed to keep the bound denatured proteins in soluble complexes as observed in the previous study [9], most likely due to lacking the second step binding and the assistance from other structural components. On the other hand, the rest region of Hsp16.3 lacking the first binding site was unable to bind denatured proteins at all [9], most likely due to lacking the first step binding (predominant) and/or the limited ability of the second binding.

Interestingly, sequence alignment showed that the bis-ANS binding sites of Hsp16.3 are similar to those hydrophobic probe and/or substrate protein binding sites of sHSPs identified so far (as summarized in Fig. 3), which are exclusively located in the N-terminal region and in the N-terminus of α-crystallin domain. This observation suggests that these two regions in sHSPs are functionally conserved as substrate-binding sites although their primary sequences show variations, particularly the N-terminal region. However, obvious differences are also presenting between these two sites in sHSPs. The N-terminal region indeed shows higher variation and hydrophobicity in primary sequence, and more disorder in secondary structures than the N-terminus of α-crystallin domain does [2,5,7,9,23,24]. Such differences in structural properties will allow the former one to have higher structural flexibility, and wider specificity and stronger interaction to denatured proteins [18,25]. Furthermore, the former one is mainly involved in the high level of oligomerization while the latter one mainly in dimerization [7,24], and this difference will lead the former one to be exposed more easily during the oligomeric dissociation of sHSPs. Together, these differences suggest that the N-terminal region might play more important roles than the N-terminus of α-crystallin domain in recognizing and/or binding denatured substrate proteins. Therefore, the two-step binding hypothesis to Hsp16.3 might be applicable to the sHSPs protein family. Furthermore, this hypothesis might provide some implications into understanding the releasing pathway of substrate proteins from sHSPs–substrate complexes, in which substrate proteins were found to be released under the help of other chaperone systems like Hsp70 [26].

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