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Small heat-shock proteins function in the insoluble protein complex ☆

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Abstract

Small heat-shock proteins (sHSPs) represent an abundant and ubiquitous family of molecular chaperones. The current model proposes that sHSPs function to prevent irreversible aggregation of non-native proteins by forming soluble complex. The chaperone activity of sHSPs is usually determined by the capacity to suppress thermally or chemically induced protein aggregation. However, sHSPs were frequently found in the insoluble complex particularly in vivo. In this report, it is clearly revealed that the insoluble sHSP/substrate complex is formed when sHSP is overloaded with non-native substrates, which is the very case under in vivo conditions. The proposal that sHSPs function to prevent the protein aggregation seems misleading. sHSPs appear to promote the elimination of protein aggregates by incorporating into the insoluble protein complex.

Keywords: Small heat-shock protein; Molecular chaperone; Chaperone activity; Protein aggregation; Insoluble complex

Since exposure to high temperature represents a serious threat to cellular viability, organisms have developed a response mechanism by synthesizing a group of proteins among which mostly are molecular chaperones, assisting other cellular proteins to function correctly. Small heat-shock proteins (sHSPs) represent an abundant and ubiquitous family of molecular chaperones. In contrast to other chaperone families (e.g., Hsp100, Hsp90, Hsp70, and Hsp60), the sHSP family is characterized by having a conserved "α-crystallin domain" and a low molecular mass per subunit (~12–42 kDa) [1–4].

The protein aggregates were toxic to the cells since they would impair the normal cellular functions [5]. According to the current model, sHSPs are proposed to prevent the irreversible protein aggregation and insolubilization under stress conditions, by binding these non-native proteins to form soluble complex [6–10]. The in vitro chaperone activities of sHSPs are therefore usually determined by their capacity to suppress thermally or chemically induced aggregation of some model proteins [8,9]. Enormous efforts have been made to elucidate the associated mechanisms of sHSPs [1,2]. In contrast to the current model, sHSPs were frequently found to localize in the insoluble fractions incorporated with the non-native proteins, particularly under in vivo conditions (e.g., IbpA/B [11–13], Hsp26 [14,15], Hsp16.6 [16], and Hsp27 [17]). In view of the nature of sHSPs themselves to be highly resistant to thermal aggregation[8,18,19], it is not unreasonable to assume that sHSPs function not simply via forming the soluble complex. This study was performed to explore this "soluble/insoluble" paradox, using the *Escherichia coli* sHSP IbpB.

IbpB together with IbpA was originally identified from the inclusion bodies, the protein aggregates formed when certain recombinant proteins are overexpressed

☆ *Abbreviations:* sHSP, small heat-shock protein; IbpB, inclusion-body binding protein B; DTT, 1,4-dithiothreitol.

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[11]. They both belong to the sHSP family and share 50% similarity. As other members of sHSPs, IbpB was shown to be highly induced upon the heat-shock temperatures and exhibit in vitro chaperone activity [18,20]. In this report, by analyzing the solubility of IbpB during displaying chaperone activity, it is found that the state of IbpB–substrate complex is mainly dependent on the IbpB/substrate stoichiometric ratio. IbpB enters into the precipitate when it is overloaded with the non-native substrates, and this is the very case of the in vivo conditions for *E. coli* cells, where IbpB is mainly localized in the insoluble fractions. The protein aggregates where IbpB is involved are easier to be eliminated, which appears to be the consequence of specific interactions of sHSPs with other chaperones.

Materials and methods

Materials. IbpB protein is purified as previously described [18]. The rabbit antiserum against IbpB was generated according to the protocol [21]. *E. coli* strain of MC4100 Δ*ibp* is a generous gift from F. Baneyx of Washington University [22]. Insulin, alcohol dehydrogenase, and malate dehydrogenase were purchased from Sigma (USA). All other chemical reagents were of analytical purity.

Assay of chaperone activity of IbpB in vitro. The chaperone activity was assayed according to previously described [6,18,19], by measuring the capacity of IbpB proteins to suppress the DTT-induced aggregation of insulin B chains at 45 °C, which was monitored at 360 nm with a spectrophotometer (Amersham Biosciences).

Analytical size-exclusion chromatography. The size-exclusion chromatography was performed on an AKTA Purifier operation system (Amersham Biosciences) using Superose 6 HR 10/300 column with the Phosphate Buffer as the eluent (50 mM Na_3PO_4, 50 mM NaCl, pH 7.3). The flow rate was 0.4 mL/min. The samples were centrifuged at 10,000*g* for 10 min before each loading.

Solubility analysis of IbpB in the E. coli lysate. The solubility of IbpB in *E. coli* MC4100 cells was assessed with 500 mL shake flasks containing 300 mL LB medium supplemented with 0.1% streptomycin. Water bath was used for culturing cells at the indicated temperatures (30 or 45 °C). The cells were grown at 30 °C to mid-exponential phase ($OD_{600} \sim 0.4$), immediately shifted to 45 °C for 30 min and then transferred back to 30 °C for recovery. The separation of different fractions of the cells was mainly performed as described previously [23]. Briefly, 40 mL aliquots of culture were taken out and cooled on the ice at indicated time points shown in Fig. 3A. Then the cells were collected by centrifugation at 5000*g* for 10 min at 4 °C, suspended in 100 μL buffer A (10 mM K_3PO_4, 1 mM EDTA, pH6.5, plus 20% sucrose and 1 mg/mL lysozyme), and incubated on ice for 30 min. The cell lysis was performed by adding 900 μL buffer B (10 mM K_3PO_4, 1 mM EDTA, pH6.5), followed by ultrasonication while cooling. Intact cells were removed by centrifugation at 2000*g* for 15 min at 4 °C. The soluble fraction was isolated by subsequent centrifugation at 15,000*g* for 20 min at 4 °C. The pellet fractions were washed with the buffer B and suspended in the corresponding volume as the supernatant. The samples with identical amount of the total, soluble, and insoluble fractions were loaded onto the Tricine–SDS–PAGE for analysis [24]. After the electrophoresis, the proteins from the gels were transferred to one membrane for Western blotting analysis.

Separation of the protein aggregates of E. coli cells. The separation of the protein aggregates of wild-type and Δ*ibp* MC4100 *E. coli* cells was mainly performed as previously described [23]. The insoluble fractions of the cells at different treatment stages (as indicated in Fig. 3B) were separated by the same method illustrated above, and furthermore, the membrane proteins in such insoluble fractions were washed away with buffer B containing 2% NP40. The corresponding protein aggregates were finally suspended in 100 μL buffer B and analyzed by Tricine–SDS–PAGE.

Results

The solubility of sHSP–substrate complex depends on the sHSP/substrate ratio

sHSPs were proposed to prevent irreversible protein aggregation and insolubilization [6–10]. In view of the paradox that some sHSPs were frequently found to localize in the thermally induced protein pellets [11–17,25,26], the solubility of the small heat-shock protein IbpB during the chaperone activity is thus investigated in detail here. The chaperone activity is usually measured in vitro by the capacity to suppress the aggregation of the insulin B-chain induced by DTT reduction of the disulfide bonds. As the data presented in Fig. 1A, IbpB can indeed prevent the aggregation of insulin B-chain in a dose-dependent manner: with the increase of IbpB concentration, the degree to suppress the insulin aggregation is increased. Whereas, once the protein aggregation of insulin is not fully suppressed, most IbpB enters into

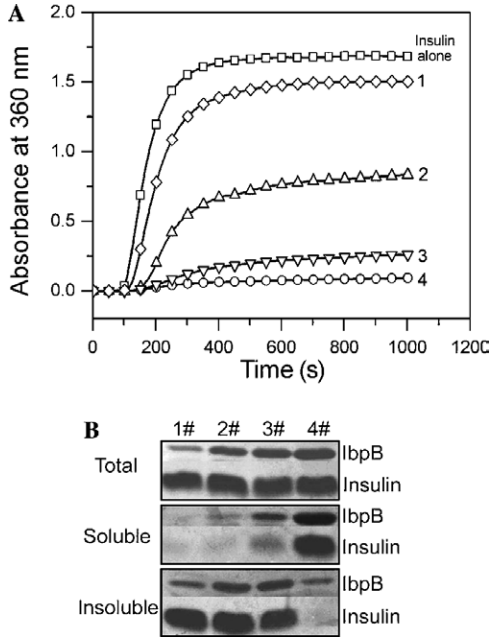

Fig. 1. Analysis of the IbpB solubility during the chaperone activity in vitro. (A) The absorbance curves of the DTT-induced insulin B chain aggregation performed at 45 °C (DTT, 20 mM; insulin, 0.5 mg/mL) in the presence of wild-type IbpB (with the final concentration of 0.025, 0.06, 0.125, and 0.2 mg/mL for curve 1, 2, 3, and 4, respectively). (B) SDS–PAGE analysis results of the total, soluble, and insoluble fractions of the samples (1–4 as described in A) during chaperone activity.

the insoluble fraction (see lanes 1–3, Fig. 1B). The similar phenomena are found when other sHSPs such as α-crystallin and Hsp16.3, or other model substrates such as alcohol dehydrogenase, malate dehydrogenase, and *E. coli* cell extracts are used (data not shown).

It is clearly suggested that IbpB remains soluble only when the substrate aggregation is completely suppressed (4# in Figs. 1A and B). In such a case, as analyzed by size-exclusion chromatography and SDS–PAGE (1# in Fig. 2), most IbpB form the soluble complex incorporated with the substrate. It is subsequently asked whether such soluble complex can be converted into the insoluble one when provided with more substrates. As the data presented in Fig. 2 show (from 1# to 5#), the soluble IbpB–insulin complexes are progressively converted into insoluble pellets with the addition of substrates. Interestingly, the soluble complexes can still exhibit some chaperone activity like the IbpB proteins (Fig. 2A), it suggests the binding stoichiometry between IbpB/substrate is not constant. Taken together, the solubility of IbpB–substrate complex is mainly dependent on the IbpB/substrate stoichiometry. The insoluble complex is formed when excessive substrate is provided relative to IbpB.

IbpB exists mainly in the insoluble fraction in vivo to assist the elimination of the protein aggregates

It is subsequently asked IbpB exists as soluble or insoluble under the in vivo conditions. The data presented in Fig. 3A show the Western blotting results, with the antiserum against IbpB, of the fractions of *E. coli* cells during the different stages of heat shock and recovery. Little IbpB is detected under normal growth temperature (30 °C, lane 1, Fig. 3A), but it is greatly induced by the heat-shock treatment at 45 °C (sub-lethal temperature for *E. coli*) for 30 min (lane 2, Fig. 3A). Intriguingly, IbpB is found to exist mainly in the insoluble fraction (compare the soluble and insoluble fractions in lane 2, Fig. 3A). After the recovery at 30 °C for a period of time, IbpB in both the whole cell and the insoluble fractions progressively disappears (from lane 2 to 6, Fig. 3A), while the amount of IbpB in the soluble fractions remains relatively constant until the IbpB in the insoluble fractions totally disappears. Thus, the function of IbpB appears to be highly restricted to the heat-shock conditions by incorporating into the insoluble fraction of *E. coli* cells.

The corresponding insoluble protein aggregate of the wild-type and Δ*ibpAB E. coli* cells at the different stages are separated and analyzed by Tricine–SDS–PAGE (Fig. 3B). It is also clearly indicated that the IbpA/B are incorporated into the protein aggregates and are greatly overwhelmed by the associated aggregate proteins (see lane 6 in Fig. 3B). Furthermore, the heat-shock induced protein aggregates of the wild-type cells

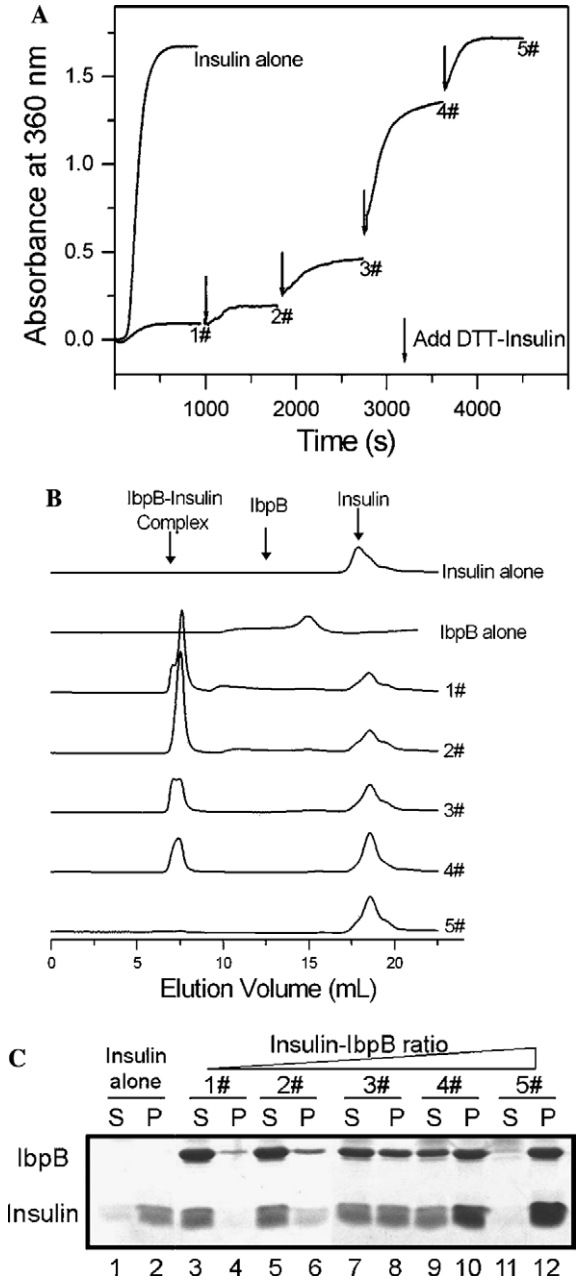

Fig. 2. Soluble IbpB–substrate complex can be converted into insoluble one provided with overloaded non-native substrates. (A) The absorbance curves of the DTT-induced insulin B chain aggregation performed at 45 °C in the presence of IbpB (with the final concentration of 0.2 mg/mL), and the DTT and insulin with the ascending amount. At the time point after every 900 s (marked with a downward arrow), the DTT and insulin with the final concentration of 20 mM and 0.5 mg/mL, respectively, were progressively added into the reaction samples. The samples (as labeled by 1# to 5#) were collected for the analysis by size-exclusion chromatography and SDS–PAGE. (B) The size-exclusion chromatography analysis curves of the samples 1# to 5# with the ratio of insulin/IbpB progressively increased as described in A. The curves of insulin and IbpB alone are also shown. Marked on the top are the elution positions of IbpB–insulin complex, IbpB, and insulin, respectively. (C) SDS–PAGE analysis results of the soluble and insoluble fractions of the samples 1# to 5# with insulin–IbpB ratio progressively increased as described in A.

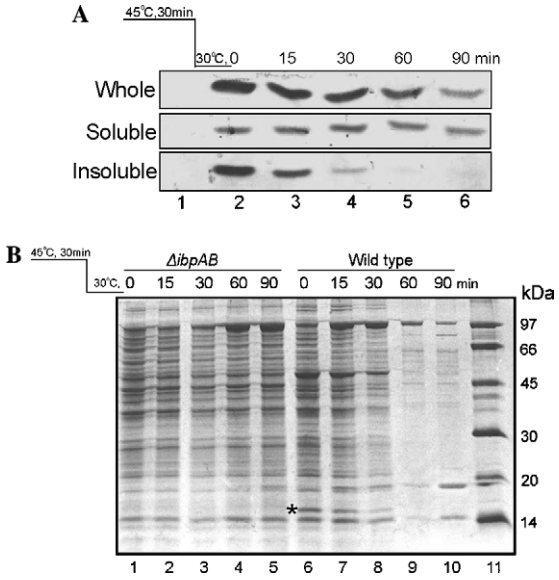

Fig. 3. Analysis of IbpB solubility in vivo and the insoluble protein aggregates separated from *E. coli* cells. (A) The Western blotting results of whole, soluble, and insoluble fractions separated from *E. coli* cells with the antiserum against IbpB. The cells are cultured to mid-exponential phase (lane 1), shifted to 45 °C for 30 min (lane 2), and then transferred to 30 °C for recovery (15, 30, 60, and 90 min for lanes 3–6, respectively). Equal amount of fractions was loaded. (B) The Tricine–SDS–PAGE analysis results of the insoluble protein aggregates separated from the wild-type and Δ*ibpAB E. coli* cells during the recovery phases at 30 °C from the heat shock treatment at 45 °C for 30 min. The cells are cultured to mid-exponential phase, shifted to 45 °C for 30 min, transferred to 30 °C for recovery for the indicated time, and then centrifuged to separate the insoluble protein aggregate. The asterisk marks the position of IbpA/B (16 kDa). Right-hand side show the molecular standard markers.

are shown to gradually disappear, while those from the mutant are not (lanes 1–5 for Δ*ibpAB*, and lanes 6–10 for WT, Fig. 3B).

Discussion

Many physical or chemical factors may cause the formation of protein aggregates in cells, the elimination of which seems important for the organisms since they would be toxic to cells and impair the cellular function, and even render some neurodegenerative diseases in animals [5,27]. sHSPs were proposed to prevent the protein aggregation by forming the soluble complex with the aggregation-prone substrate proteins. It is clearly suggested here that the soluble complex can be converted into the insoluble one when provided with excessive substrates, and under the in vivo conditions, the sHSPs are indeed overloaded with the non-native substrate proteins and most of sHSPs exist in the insoluble complex. The incorporation of sHSPs into the insoluble complex can assist the elimination of the protein aggregates. All these data together suggest that the major function of sHSPs is not the suppression of protein aggregation as it was shown before but their ability to incorporate into the protein aggregate.

Recently, sHSPs are demonstrated to form a network together with Hsp70 and Hsp100 to efficiently refold the non-native proteins from the protein aggregates [14,15,28,29]. Hsp70 alone was shown to be able to mediate the refolding of substrates from the soluble complex, whereas Hsp100 was essential for the refolding of substrates from the insoluble complex [14]. Meanwhile, it was found that Hsp100 was critical for the thermal tolerance of yeast or bacterial cells [22,30,31]. It suggests that, upon the heat-shock stress, the insoluble protein aggregates indeed exist and the elimination of such protein aggregates by the multi-chaperone network is critical for the viability of cells. The presence of sHSPs in protein aggregates may represent the evolutionary income of organisms to efficiently remove such toxic factors.

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