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Assessment of substrate-stabilizing factors for DnaK on the folding of aggregation-prone proteins

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ABSTRACT

Hydrophobic interactions between molecular chaperones and their nonnative substrates have been believed to be mainly responsible for both substrate recognition and stabilization against aggregation. However, the hydrophobic contact area between DnaK and its substrate proteins is very limited and other factors of DnaK for the substrate stabilization could not be excluded. Here, we covalently fused DnaK to the N-termini of aggregation-prone proteins *in vivo*. In the context of a fusion protein, DnaK has the ability to efficiently solubilize its linked proteins. The point mutation of the residue of DnaK critical for the substrate recognition and the deletion of the C-terminal substrate-binding domain did not have significant effect on the solubilizing ability of DnaK. The results imply that other factors of DnaK, distinct from the hydrophobic shielding of folding intermediates, also contributes to stabilization of its noncovalently bound substrates against aggregation. Elucidation of the nature of these factors would further enhance our understanding of the substrate stabilization of DnaK for expedited protein folding.

The hydrophobic interaction-mediated substrate recognition and stabilization against aggregation have provided a conceptual framework for the understanding of protein folding assisted by molecular chaperones [1,2]. However, most proteins fold without the assistance of the known molecular chaperones in the *Escherichia coli* cytosol [3,4], suggesting the existence of other chaperoning mechanisms. A representative molecular chaperone DnaK, an *E. coli* Hsp70 homolog, preferentially recognizes short linear peptides with 2–4 exposed consecutive hydrophobic residues flanked by basic residues in protein sequences [5,6]. In contrast, BiP, Hsp70 homolog in the endoplasmic reticulum, can bind the hydrophilic peptides without hydrophobic amino acids [7]. Moreover, electrostatic interactions were suggested to play an important role in the substrate recognition of GroEL/pepsin [8], α-crystallin/fibroblast growth factor-1 [9], and eukaryotic type II chaperonin (TCP-1)/actin [10]. In addition, the substrate recognition of calnexin and calreticulin is mainly mediated through the binding to the glycan moiety [11]. These phenomena of non-hydrophobic interaction-mediated substrate recognition cannot be easily explained by the hydrophobic interaction-mediated chaperoning mechanism.

The obvious charge effects on protein solubility have been well established, as shown in close correlation between net charge and solubility of proteins [12], repeated charged residues as structural gatekeepers in protein sequences [13], and the solubility maintenance of natively unfolded proteins under the physiological conditions [14]. The net charge of fusion tags are also an important parameter for solubilizing their linked aggregation-prone proteins [15,16]. The electrostatic repulsions by charged residues were suggested to prevent protein aggregation [17]. In addition, the steric hindrance of glycan moiety and polyethylene glycol (PEG) was reported to inhibit aggregation of their linked proteins [18,19]. We previously reported that the electrostatic repulsions and steric hindrance of folded N-terminal domains could solubilize their downstream domains, contributing to the autonomous folding of multidomain proteins *in vivo* [20]. The growing evidence strongly indicates that the hydrophobic shielding by direct contact is not a sole determinant factor for the stabilization of aggregation-prone polypeptides against aggregation in the aqueous environments.

The substrate stabilization by the bound chaperones against aggregation is their highlight function. So far, it has not yet been proven experimentally whether the limited hydrophobic

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interactions between DnaK and its substrate proteins are mainly responsible for the substrate stabilization against aggregation. The importance of hydrophobic interactions for the substrate recognition of DnaK makes it extremely difficult to independently assess the role of these interactions in the substrate stabilization against aggregation. To overcome this problem, here we covalently fused aggregation-prone proteins to the C-termini of DnaK and its variants with impaired substrate recognition and investigated the solubilizing ability of DnaK in the *E. coli* cytosol. The *cis*-acting solubilizing ability of DnaK was not significantly influenced by its hydrophobic interaction-mediated substrate recognition ability that functions in a *trans*-acting mode. Our results indicate that DnaK has an ability to solubilize its linked aggregation-prone proteins irrespective of its hydrophobic interaction-mediated substrate recognition ability.

Materials and methods

Expression vector construction. The expression vectors, derived from pGEMEX-1 (Promega), carrying human granulocyte colony-stimulating factor (GCSF), *Aquorea victoria* green fluorescence protein (GFP), and tobacco etch virus protease (TEVP) in the unfused form were adopted from our previous report [20]. In the same context, the genes encoding DnaK and its variants-fused proteins were cloned. The genes of DnaK and its N-terminal domain, DnaK(N), encoding the 1–384 residues of DnaK, were obtained from the *E. coli* genomic DNA through PCR amplification. A DnaK mutant, DnaK(V436F), was constructed by overlapping PCR mutagenesis. The linker sequence of GTGSSTSTST was used between the N-terminal fusion partners (DnaK, DnaK(V436F), and DnaK(N)) and passenger proteins (GCSF, GFP, and TEVP).

Protein expression and solubility test. The *E. coli* strain of HMS174(DE3) plysE (Novagen) was used as a expression host. The HMS174(DE3) plysE harboring each expression vector were cultured at 37 °C. At the cell density (A_{600}) of 0.5–0.7, IPTG was added to a final concentration of 1 mM for the protein expression. After 3-h culture at 37 °C, the culture broth were harvested by centrifugation and stored at −70 °C until analysis. The plasmid encoding DnaK-DnaJ-GrpE [21] was kindly provided from O. Fayet (Laboratoire de Microbiologie et Génétique Moléculaires, France). The HMS174(DE3) harboring DnaK-DnaJ-GrpE expression vector and each GCSF, GFP, and TEVP expression vector were cultured at 37 °C. The expression of the chaperone teams were induced with the treatments of 0.02% L-arabinose. The solubility of target proteins was estimated on SDS–PAGE with gel densitometer (Vilber Loumat) as described previously [20].

Protein purification and TEVP activity assay. The His-tagged TEVP fusion proteins (DnaK–TEVP, DnaK(V436F)–TEVP, and DnaK(N)–TEVP) and MBP-GCSF harboring TEVP cleavage site were purified on Ni-affinity chromatography. The concentrations of purified TEVP fusion proteins and their substrate MBP-GCSF were determined by BCA method (Pierce). The TEVP cleavage reaction was carried out in 100 μL volume contains 50 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT, 10 μg MBP-GCSF as substrate, and 8 U rTEV (Invitrogen) or 0.3 μM of each TEVP fusion protein for 2 h at 30 °C. The cleavage reaction mixtures were analyzed by SDS–PAGE.

Results

DnaK can efficiently solubilize its C-terminal aggregation-prone proteins in vivo

To assess the factors of DnaK responsible for stabilizing its noncovalently bound substrates against aggregation, we assumed that the DnaK fusion protein in the folding intermediate state, where DnaK is folded while the C-terminal passenger protein is not folded, could mimic the noncovalent complex of DnaK and its aggregation-prone substrate protein. This approach enabled us to investigate the solubilizing ability of DnaK and its correlation with the hydrophobic interaction-mediated substrate recognition ability of DnaK in a *trans*-acting mode.

DnaK was fused to the N-termini of three aggregation-prone passenger proteins including GCSF, GFP, and TEVP. When directly expressed without fusion, all three tested proteins were expressed predominantly as inclusion bodies at 37 °C (Fig. 1A). After fusion, however, DnaK dramatically increased the solubility of its linked proteins expressed at 37 °C; the solubilities of DnaK–GCSF, DnaK–GFP, and DnaK–TEVP were approximately 79%, 77%, and 47%, respectively (Fig. 1B). These results indicate that DnaK exhibits the ability to efficiently solubilize covalently linked aggregation-prone proteins *in vivo*.

However, distinct from the conventional solubility enhancers such as *E. coli* maltose binding protein and thioredoxin, DnaK can function as chaperone in a *trans*-acting mode. Thus, it is possible that the *trans*-acting chaperone activity of the fused DnaK still contributes to its solubilizing ability for the overexpressed DnaK fusion proteins. We investigated the effect of coexpression of DnaK-DnaJ-GrpE chaperone teams on the solubility of the above three passenger proteins in the unfused form. The coexpression of DnaK-DnaJ-GrpE had little or no effect on the solubility of GCSF and TEVP whereas it increased the solubility (approximately 15%) of GFP significantly (Fig. 1C). Overall, the solubility enhancement by the coexpression of DnaK chaperone team was marginal as compared with the DnaK fusion. Previously, the coexpression of chaperones has been attempted and proven effective only in the very limited heterologous proteins [22]. The results in Fig. 1A–C are now summarized in Fig. 1D, suggesting that the *cis*-acting solubilizing ability of DnaK may not result from its previously known *trans*-acting chaperoning activity.

The residue and domain critical for the substrate recognition of DnaK do not have a significant impact on the cis-acting solubilizing ability of DnaK

Here, we investigated the *cis*-acting solubilizing ability of DnaK mutants with severely impaired substrate-binding affinity. Previously, the DnaK(V436F) with valine changed into phenylalanine at residue 436 in the hydrophobic pocket of its C-terminal substrate-binding domain, has been reported to lose the chaperoning activity both *in vitro* and *in vivo* [23,24]. The DnaK(V436F) was fused to the N-termini of three test proteins. As shown in Fig. 2A, the DnaK(V436F) fusion proteins exhibited the solubility similar to that of their corresponding DnaK(wt) fusion proteins at 37 °C (approximately 68%, 64%, and 44% for DnaK(V436F)–GCSF, DnaK(V436F)–GFP, and DnaK(V436F)–TEVP, respectively). As compared with the corresponding DnaK(wt) fusion proteins, the solubilities of DnaK(V436F)–GCSF, DnaK(V436F)–GFP was slightly decreased whereas the solubility of DnaK(V436F)–TEVP was not changed. The results indicate that the *cis*-acting solubilizing ability of DnaK does not significantly depend on its residue critical for the substrate recognition in a *trans*-acting mode.

Although DnaK(V436F) loses its chaperone activity in a *trans*-acting mode to a critical extent, it cannot be completely ruled out that the residual substrate-binding affinity of DnaK(V436F) might still contribute to the *cis*-acting solubilizing ability of DnaK(V436F) via transient hydrophobic interactions with its C-terminal proteins in an intramolecular manner. To test this, the DnaK(N), the N-terminal ATPase domain of DnaK without the C-terminal substrate-binding domain, was fused to three passenger proteins. As shown in Fig. 2B, the N-terminal domain alone exhibited the solubilizing ability as similar to DnaK and DnaK(V436F) (99%, 99%, and 23% for DnaK(N)–GCSF, DnaK(N)–GFP, and

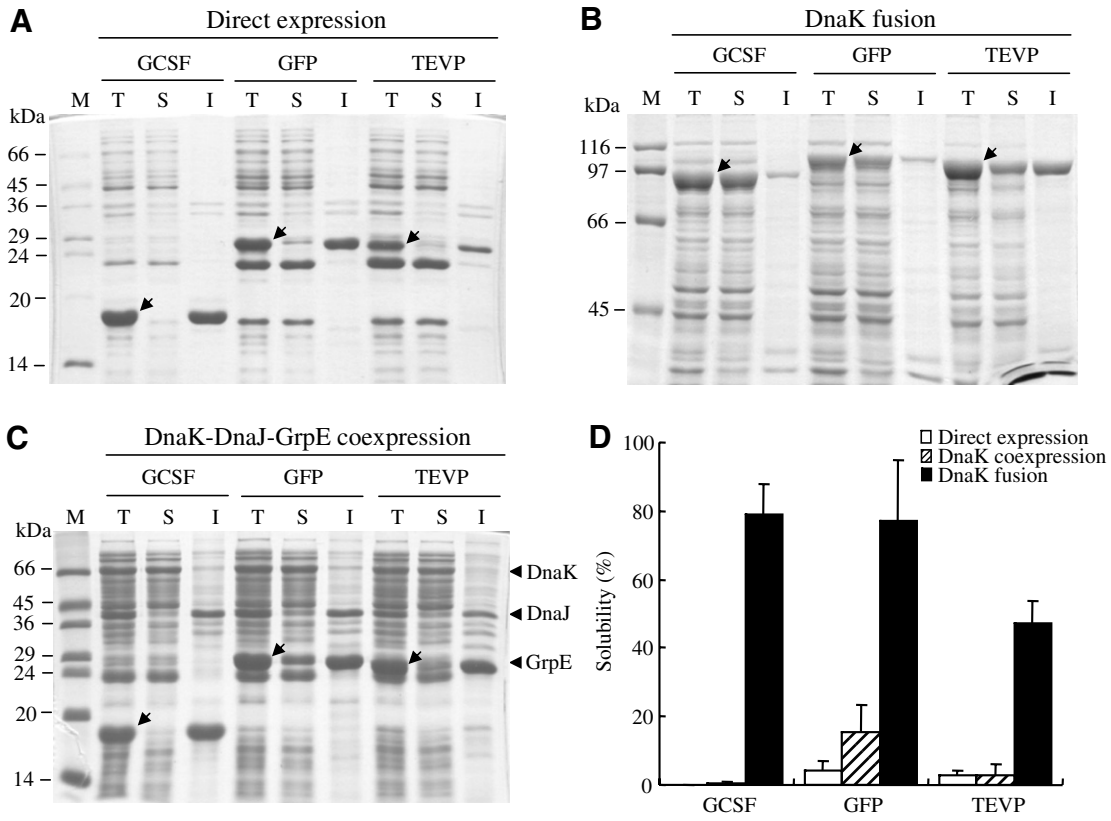

Fig. 1. The solubilizing ability of DnaK for its C-terminal aggregation-prone proteins *in vivo*. (A) Direct expression of aggregation-prone proteins. (B) The expression of DnaK fusion proteins. DnaK was fused to the N-termini of three aggregation-prone proteins (GCSF, GFP, and TEVP). (C) The effect of coexpression of DnaK-DnaJ-GrpE chaperone team on the solubility of directly expressed proteins. (D) Summaries on the solubility of tested proteins in A–C. The proteins were expressed at 37 °C and analyzed by SDS–PAGE. Throughout the whole figures, the measurement of solubility was from three independent experiments, and error bars represent the standard deviations. T, S, and I indicate total lysates, soluble fraction, and insoluble fraction, respectively.

DnaK(N)–TEVP, respectively). Compared with DnaK or DnaK(V436F) fusion proteins, the solubilities of DnaK(N)–GCSF and DnaK(N)–GFP were even higher whereas that of DnaK(N)–TEVP was lower. The overall results in Figs. 1 and 2 strongly suggest that DnaK have the solubilizing ability to its linked aggregation-prone proteins, and that this ability of DnaK does not depend on its hydrophobic interaction-mediated substrate recognition. Importantly, our results suggest that other factors of DnaK, distinct from the direct hydrophobic shielding, might be important for stabilizing its noncovalently bound substrates against aggregation.

DnaK and its variants promote the proper folding of its downstream proteins in the fusion context

To investigate if the C-terminal passenger proteins reach their proper folding in the fusion context, the functional activity of the fusion proteins was examined. For this purpose, the TEVP fusion proteins, including DnaK–TEVP, DnaK(V436F)–TEVP, and DnaK(N)–TEVP, were purified on nickel affinity chromatography (Fig. 3A), and the cleavage of the substrate protein MBP-GCSF harboring TEVP recognition site was examined. As shown in Fig. 3B, all TEVP fusion proteins specifically cleaved MBP-GCSF into two fragments of expected size. The results indicate that DnaK can promote the proper folding as well as solubility of their C-terminal proteins *in vivo* irrespective of the hydrophobic interactions crucial for the substrate recognition in a *trans*-acting mode.

Discussion

The present results indicate that the solubilizing ability of DnaK, in the context of a fusion protein, does not depend on its well-known hydrophobic interaction-mediated substrate recognition ability. A close proximity of substrate protein to DnaK via covalent linkage was found to be sufficient for the observed solubility and folding enhancement. Generally molecular chaperones as well as solubility enhancers assist protein folding in a passive manner by preventing protein aggregation [20,25–27]. The *cis*-acting solubilizing mechanism of DnaK is likely similar to that of the commonly used solubility enhancers. Accordingly, the *cis*-acting solubility increase and subsequent folding enhancement in a fusion context may have relevance to the chaperone activity of DnaK that normally functions in a *trans*-acting manner. It is conceivable that irrespective of the limited hydrophobic interactions between DnaK and its substrates, DnaK might have an intrinsic ability to stabilize its noncovalently bound substrates against aggregation. The current prevailing concept of the hydrophobic interaction-mediated substrate stabilization by the bound chaperones does not include the effects of the properties of chaperones such as their size, shape or surface charge that would also contribute to substrate stabilization.

How could DnaK stabilize its bound substrates against aggregation even without significant hydrophobic shielding? To explain the present observation, a simplified model is presented in Fig. 4. A soluble macromolecule (sphere ***A***) such as DnaK with varying radius r and constant surface charge density interacts, via

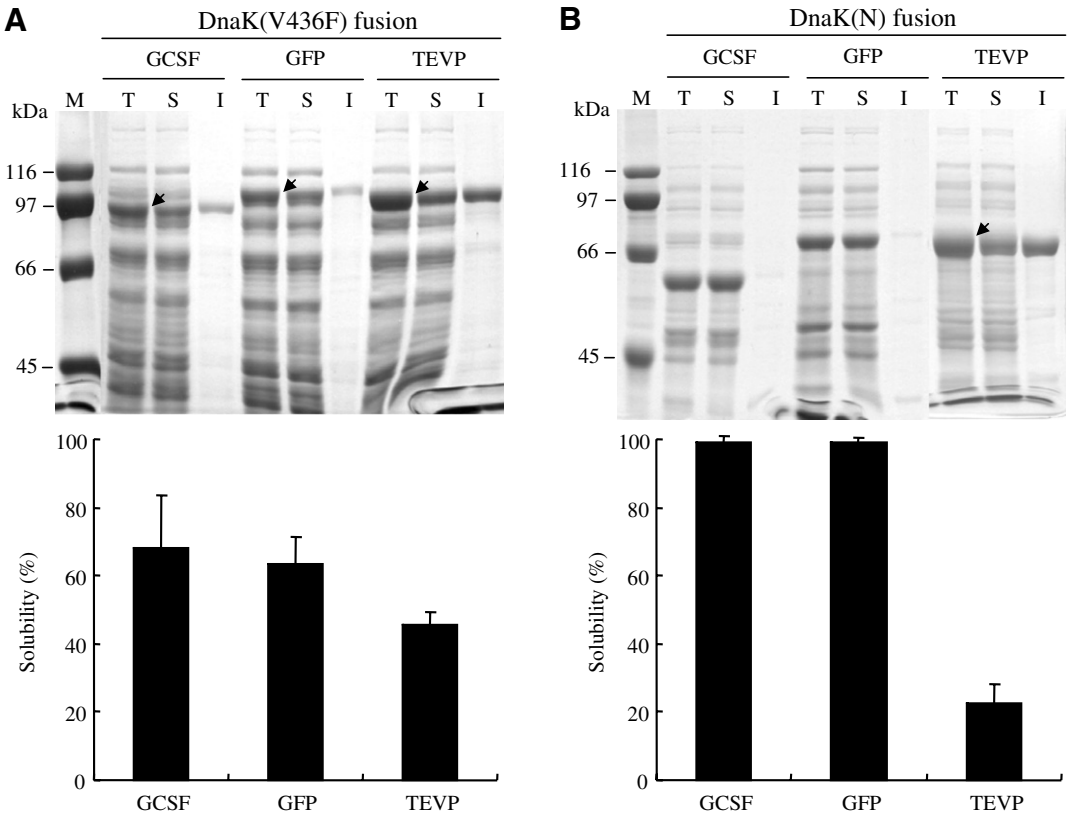

Fig. 2. The solubilizing ability of DnaK mutants with impaired substrate-binding ability. (A) The solubilizing ability of DnaK(V436F). (B) The solubilizing ability of the N-terminal domain of DnaK, DnaK(N). To investigate the effect of hydrophobic interaction-mediated substrate recognition of DnaK on its solubilizing ability, DnaK(V436F) or DnaK(N) were fused to the N-termini of three proteins (GCSF, GFP, and TEVP). The fusion proteins expressed at 37 °C were analyzed by SDS–PAGE.

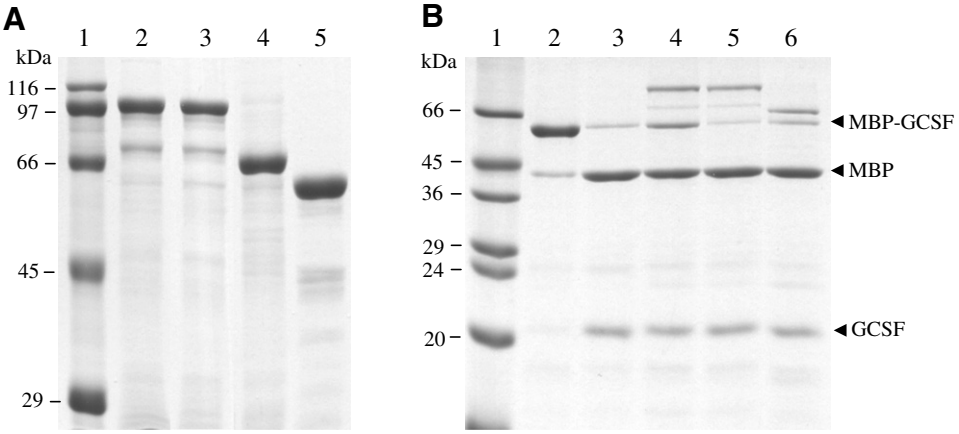

Fig. 3. The functional activity of DnaK fusion protein. (A) Purified TEVP fusion proteins and their substrate protein MBP-GCSF. The DnaK- or its variants-fused TEVP and MBP-GCSF were purified on Ni-affinity chromatography. Lane 1: size marker, lane 2: DnaK–TEVP, lane 3: DnaK(V436F)–TEVP, lane 4: DnaK(N)–TEVP, lane 5: MBP-GCSF. (B) *In vitro* TEVP activity assay. The MBP-GCSF was treated with TEVP fusion proteins. Lane 1: size marker, lane 2: MBP-GCSF only, lane 3: MBP-GCSF + rTEV (Invitrogen), lane 4: MBP-GCSF + DnaK–TEVP, lane 5: MBP-GCSF + DnaK(V436F)–TEVP, lane 6: MBP-GCSF + DnaK(N)–TEVP. The cleavage reactions were analyzed by SDS–PAGE.

limited hydrophobic interactions, with an aggregation-prone protein (sphere ***B***). From steric point of view, the excluded volume of ***A*** is not penetrable by other molecules in an intermolecular association process, thereby preventing aggregation driven by ***B***; especially the excluded volume repulsion of ***A*** masks the surface of ***B*** that is in close proximity to ***A***, rendering the surface inaccessible to other ***B*** molecules that would otherwise lead to intermolecular aggregation. The total surface charge that correlates with electrostatic repulsion and the excluded volume of ***A*** are proportional to r^2 and r^3, respectively. In contrast, the hydrophobic interactions between ***A*** and ***B*** is nearly constant and insensitive to r. Therefore, depending on the size of ***A***, there is a variable contribution of the charge and steric factors provided by ***A*** to the stabilization of ***B***, which could be even greater than that provided by hydrophobic interaction. Importantly, the contributions of these factors would not be affected significantly by the replacement of the usual hydrophobic interactions between ***A*** and ***B*** with the covalent linkage. The substrate-stabilizing effect that would be provided in close association between ***A*** and ***B*** would be similar in both cases, suggesting that the *cis*-acting folding

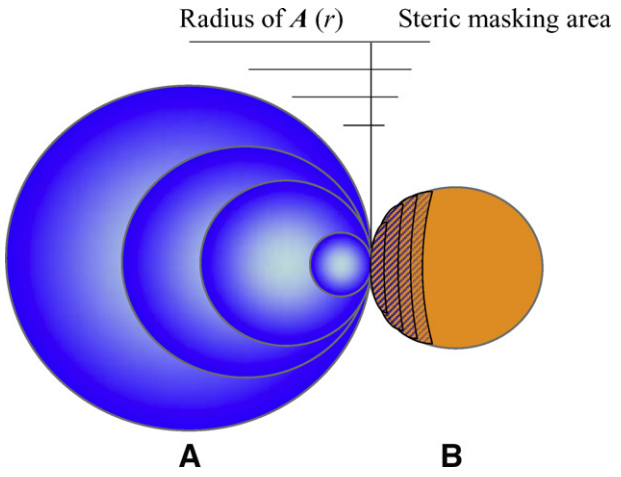

Fig. 4. A schematic illustration of potential substrate-stabilizing factors of macromolecule and their correlation with the size of macromolecule. Here, soluble macromolecule such as DnaK with varying radius r and constant surface charge density and its bound aggregation-prone protein are represented as sphere ***A*** and ***B***, respectively. The potential factors of ***A*** such as electrostatic repulsion, steric hindrance, and hydrophobic shielding are considered as a function of the radius r of ***A***. The amount of total surface charge (electrostatic repulsion) and excluded volume (steric hindrance) are proportional to r^2 and r^3, respectively, whereas the direct contact area (hydrophobic shielding) is nearly constant. The electrostatic repulsion and steric hindrance of larger ***A*** have the potential to be the dominant factors for stabilizing ***B*** against aggregation. The hatched area represents the surfaces inaccessible to other ***B*** by the steric masking of the corresponding ***A***. See text for the detailed description.

enhancement is compatible with normal *trans*-acting folding enhancement mechanism.

In the fusion context, DnaK dramatically increased the solubility of all tested proteins whereas the coexpression of DnaK-DnaJ-GrpE chaperones was not effective (Fig. 1). It is interesting to note that DnaK in the fusion context is superior to the coexpression of DnaK chaperone team under the same background of host. The dramatic enhancement of solubility of passenger proteins by DnaK fusion appears to result from several factors. First, the DnaK functions in an intramolecular manner, where the folded DnaK can persistently exert its chaperoning effect on its linked protein, different from intermolecular interaction-mediated chaperone function of DnaK on substrate proteins. Second, the covalent linkage between DnaK and the C-terminal proteins can allow the independent folding of the downstream C-terminal proteins to occur. Rapid folding is known to be an effective way to overcome the aggregation problem imposed in the macromolecular crowding condition inside the cells [28]. In contrast, the binding of DnaK to the exposed hydrophobic sites of substrates in a *trans*-manner prevents the folding of its bound substrates. Consequently, the molecular chaperones have a tendency to retard the folding rate of substrate proteins [29]. However, the ability of molecular chaperones to dissociate from the finally folded substrate proteins is a great advantage over the covalent fusion.

Our results suggest that the hydrophobic interaction-mediated substrate recognition of DnaK would not necessarily represent the sole mechanism for this interaction-driven substrate stabilization against aggregation. Consistent with the previously known charge and steric factors as mentioned earlier, these two factors of DnaK may also contribute to its substrate stabilization. Elucidation of the substrate-stabilizing factors of DnaK would further enhance our understanding of de novo folding of nascent proteins in relation to its chaperoning function. Covalent fusion as described in the present report would give new insights into how DnaK stabilize its bound substrates.

Acknowledgments

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