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Thesis Outline

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This thesis was titled “Elucidation of the mechanism by which small heat shock protein IbpA regulates its own expression at translation level”.

Background

Small heat shock proteins (sHsps), defined by conserved α-crystallin domains (ACDs) and disordered N-terminal as well as C-terminal domains (NTDs and CTDs), are members of molecular chaperones to prevent protein misfolding and aggregation. sHsps have low molecular weights of subunits (15~43 kDa) but assemble to chaperone-active dimers and different-sized oligomers. In *Escherichia coli*, there is a two-sHsp system composed of inclusion body-associated protein A (IbpA) and B (IbpB). They are highly similar in primary sequences (~50% identity) and closely collaborate, as chaperones, to form co-aggregates with denatured client proteins. However, IbpA has a unique self-suppression function at its own translation level, which is lost in IbpB (1). Oligomeric IbpA interacts with the *ibpA* 5’ untranslated region (5’ UTR) mRNA to repress its own translation process. However, the self-suppression of IbpA can be eliminated by sequestration into IbpA-substrate co-aggregates. Thus, *E. coli* IbpA can be regarded as an aggregation-sensor that self-regulates its own expression. Moreover, a recent paper reveals that IbpA also inhibits the translation of a heat shock transcription factor to downregulate the expression of many Hsps (2), which exhibits the general importance of IbpA for the heat shock response regulation. However, the mechanism is not so clear. For example, why does only IbpA possess self-suppression, but IbpB does not? This study aims to identify the structural feature(s) in IbpA that is essential for the self-regulation but is absent in IbpB and further to elucidate the detailed mechanism of IbpA self-suppression.

Experimental methods

To investigate IbpA self-suppression ability mediated by the *ibpA* 5’ UTR mRNA in vivo, a GFP reporter assay was performed. The *gfp* reporter contained the upstream

ibpA 5’ UTR sequence, by which the translation of GFP was regulated by the *ibpA* 5’ UTR. The reporter plasmids were co-transformed with other plasmids overexpressing target IbpA variant proteins.

To investigate IbpA self-suppression in vitro, a commercial reconstituted cell-free protein synthesis system PURExpress® (GeneFrontier) was used.

To visualize purified IbpA, a transmission electron microscope (TEM) was performed.

To investigate oligomerization, interaction with client proteins and interaction with the mRNA, sucrose density gradient centrifugation was conducted.

To directly investigate the mRNA-interaction ability of IbpA, a filter binding assay was performed. IbpA-mRNA mixture was applied through double membranes consisting of nitrocellulose membrane and nylon membrane. The nitrocellulose membrane was placed at upper to capture IbpA-mRNA complexes, and the nylon membrane was placed at lower to capture free mRNAs.

Results

Identification of crucial residues for IbpA self-suppression

At first, I designed *E. coli* IbpAB chimeras by replacing one domain of IbpA with the corresponding that of IbpB. The GFP reporter assay revealed that IbpA ACD was essential for the suppression function. Then I narrowed down the substitution regions among IbpA ACD and performed the GFP reporter assay to pick up the suppression-defect chimeras for one more narrowing down step. After a series of narrowing-down steps and alanine scanning, arginine 93 (R93), which was absent in IbpB, was found to be important for the IbpA self-suppression, since R93A (R93 replaced by alanine) was suppression-defect both in vivo and in vitro.

Based on the fact that R93 is conserved in γ-proteobacterial IbpAs, I demonstrated that the self-repression function of IbpA mediated by R93 is conserved in other γ-proteobacteria, like *Cedecea neteri* and *Vibrio harveyi*.

Moreover, R93 and its neighboring positively-charged residues (arginine 97 and lysine 98) are also common in human sHsps but absent in IbpB. In human sHsps, some of these conserved residues are important for secondary structure, oligomerization and chaperone function, dependent on their positive charge (3). Similarly, R97 and K98 in *E. coli* IbpA ($IbpA_{Ec}$) had charge-dependent importance for the suppression ability. However, R93 did not reply on the positive charge to maintain

the IbpA suppression function, since R93K, in which R93 was replaced with another positively-charge residue, was still suppression-defect. In order to further demonstrate the significance of R93, I mutated R93 to any other residues individually in $IbpA_{Ec}$ and all these mutants lost the suppression activity, which suggested R93 is irreplaceably important for IbpA self-regulation.

Some questions were raised – why was position 93 but not positions 97 and 98? And why only arginine? Based on the AlphaFold-predicted IbpA oligomeric structures, it was found R93 was located in a flexible loop region, while R97 and K98 were located in a stable β sheet structure. Moreover, an NTD loop region was close to R93, suggesting the potential interaction between R93 and the NTD loop region. In the NTD loop region, tyrosine 34 (Y34) was closest to R93. Further, GFP reporter assay revealed that in position 34 aromatic residues were necessary to maintain the IbpA suppression ability, which indicated the potential Y34-R93 interaction was a cation-π interaction. That might explain why only arginine but not lysine, since arginine forms stronger cation-π interactions compared to lysine.

Characterization of the IbpA-R93A mutant

In order to investigate the mechanism behind IbpA self-regulation based on R93, I studied the features of IbpA R93A mutant in detail.

Firstly, I confirmed the oligomerization ability of the IbpA-R93A mutant. It is IbpA oligomers but not dimers that interact with the *ibpA* 5' UTR mRNA (1), thus it was necessary to check the oligomeric state of the mutant. The purified $IbpA_{Ec}$-R93A tended to higher-order oligomers and longer fibrils compared to the WT, but the corresponding mutants in *C. neteri* and *V. harveyi* ($IbpA_{Cn}$-R93A and $IbpA_{Vh}$-R94A) had the same oligomerization tendencies with their individual WTs, which suggested IbpA self-regulation was not simply dependent on oligomer size.

The chaperone ability of IbpA-R93A to interact with denatured client proteins was also evaluated. The IbpA-R93A maintained the client protein-interaction ability to form smaller-size co-aggregates similar to IbpA-WT, which indicated R93 is not essential for IbpA chaperone function.

IbpA self-regulation is achieved by the interaction with the *ibpA* 5' UTR mRNA. Thus, here I also studied the mRNA-interaction ability of IbpA-R93A. The filter binding assay results showed that IbpA-R93A completely lost the interaction ability with the mRNA, which caused the suppression defect in IbpA-R93A. Moreover, IbpA-R93A

also impaired the mRNA-binding ability of IbpA-WT, suggesting the robustness of anti-suppression in IbpA-R93A. Combining the results that IbpA-R93A maintained the chaperone function, it was believed that R93-mediated self-suppression of IbpA is distinguishable from the chaperone function.

Additionally, the influence of IbpB, a paralog of IbpA, in IbpA suppression ability was also evaluated. *E. coli* IbpB ($IbpB_{Ec}$) promoted the mRNA-binding ability of $IbpA_{Ec}$-WT by forming suppression-active hetero-oligomers. However, $IbpB_{Ec}$ could not activate the inhibitory activity of $IbpA_{Ec}$-R93A even though they also formed hetero-oligomers, which further emphasized that IbpA self-regulation did not solely rely on oligomer size.

Conclusion

Overall, this thesis demonstrates that IbpA-mediated self-suppression function is conserved in γ-proteobacteria, and that the cationic residues in ACD, particularly R93, are essential for this function. Since the Arg93 mutations in IbpA ACD regions have little effect on chaperone activity, the conservation of Arg93 in γ-proteobacterial IbpAs implies that the translation suppression function would be critical for the cellular function of IbpAs, distinct from their chaperone role.

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