Biophysical analysis of intermolecular interaction between Hsc70 and DNAJB6 mediated by the G/F-linker

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Introduction

The 70kDa heat shock protein (Hsp70) is a central hub of the chaperone network for regulating various aspect of proteostasis1. Hsp70 activity is regulated by J-domain proteins (JDPs), which stimulate Hsp70 ATP hydrolysis, promoting chaperone cycle progression and modulating its interactions with downstream members of the network1–3. Many JDPs contain a Gly/Phe-rich linker (G/F-linker) involved in Hsp70 regulation through both intramolecular and intermolecular interactions4–6. In class B JDPs, this G/F-linker contains an auto-inhibitory helix that docks onto the J-domain and blocks Hsp70 binding. The substrate binding domain β (SBDβ) of Hsp70 is proposed to interact with this linker, potentially contributing to the release of auto-inhibition7. However, the molecular details of these interactions remain largely unknown. In this internship, we aim to characterize the binding interactions between Hsp70 and a class B JDP, DNAJB6, via biophysical binding assays to probe the role of the G/F-linker.

Methods

Creation of Cys mutant constructs

Polymerase chain reaction was conducted using NEB Inc. Q5® High-Fidelity 2X Master Mix on JB6 truncation constructs (residues 1-69, 1-97, 1-110) to add C-terminal Cys-containing linker (-GGSC). Mutagenesis results were confirmed by sequencing.

Expression and purification of JB6 and Hsc70

Plasmids containing His-tagged JB6-CtermCys constructs or full-length Hsc70-T204A were transformed into E. coli BL21. Cells were grown in LB and induced with IPTG. Hsc70 was purified by nickel affinity chromatography, incubated overnight with TEV protease to remove His tag, followed by a reverse affinity step and size exclusion chromatography (SEC) with a Superdex S200 10/300 Increase column (figure 1A). JB6-CtermCys were purified with an initial nickel affinity step, with an additional in-column urea wash step, cleaved overnight with TEV to remove His-tag and followed by reverse affinity purification (figure 1B). JB6- CtermCys constructs were further purified by reverse phase HPLC and lyophilized.

Fluorescent labeling of JB6

Lyophilized JB6-CtermCys constructs were reconstituted into NMR buffer containing 20 mM HEPES, 50 mM KCl, 5 mL MgCl2 with 10 mM DTT, pH 8, followed by SEC with Superdex S75 10/300 Increase column (figure 1C). Reconstituted protein was incubated with 25x fold molar excess of fluorescein-5-maleimide overnight at 4°C. Proteins were dialyzed at room temperature for three hours and subjected to SEC again to remove free label.

Fluorescence polarization anisotropy

Fluorescence polarization anisotropy (FPA) analysis was conducted on a CLARIOstar microplate reader, with instrument calibration set to 35 mili polarization units for J-domain alone. Fluorescently labeled JB6-CtermCys constructs at 500 nM was mixed with either Hsc70 or SBD β at varying concentrations and anisotropy was measured in triplicates. Hsc70 was saturated with 5 mM ATP in the FPA assays. Data with SBDβ was fitted to one-site binding model.

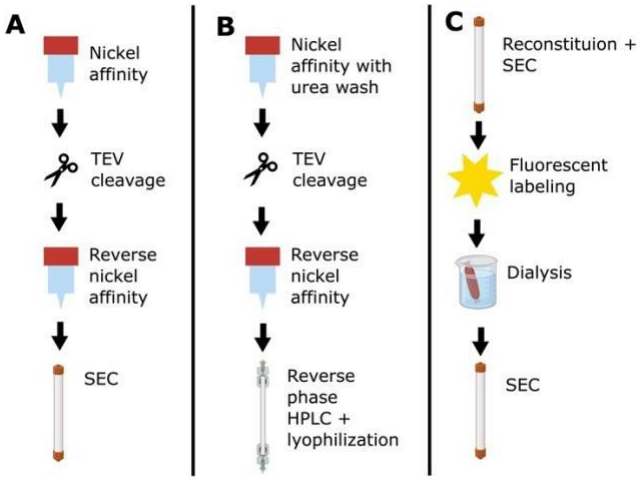

Figure 1. Protein purification schematics. A. Hsc70 purification process. **B.** JB6-CtermCys purification process. **C.** JB6-CtermCys fluorescent labeling process.

Results and Discussion

JB6-CtermCys purification for fluorescent labeling

All three JB6-CtermCys constructs were created successfully. These constructs were expressed and purified. Labeling of JB6 1-110 was successful with a monodispersed labeled protein peak observed after SEC (figure 2).

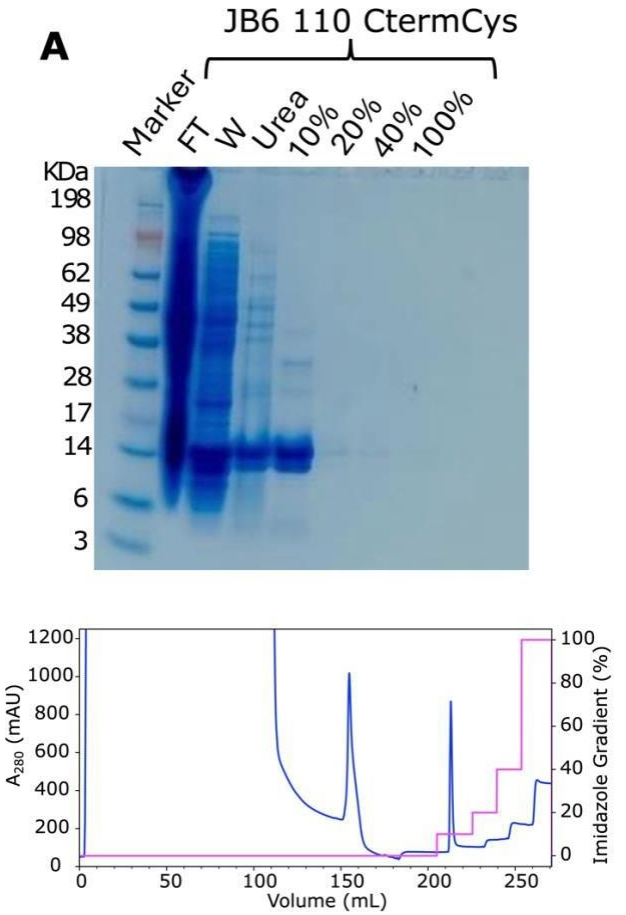

Figure 2. JB6 1-110 CtermCys was purified and fluorescently labeled. A. Nickel affinity purification of JB6 1-110 CtermCys. Upper penal: SDS-PAGE gel of different fractions (FT: flow through; W: wash; Urea: urea wash step; Percentages correspond to the imidazole gradient); Lower penal: nickel affinity chromatogram. **B.** Size exclusion chromatography (SEC) of labeled JB6 1-110 CtermCys.

Weak binding of JB6 1-110

FPA analysis were conducted between fluorescently labeled JB6 1-110 and unlabeled Hsc70 at ATP state (figure 3). A small increase in anisotropy with significant variation was observed at Hsc70 concentration range of 0-200 µM. At higher concentrations, anisotropy showed a clearer upward trend, but the absence of plateau suggests saturation was not reached.

This lack of saturation indicates low binding affinity, consistent with previous NMR data with a Kd of ~5 mM. This reflects the auto-inhibitory activity of G/F-linker on J-domain.

Hsc70 substrate binding domain β (SBDβ) has been suggested to bind G/F-linker, and was tested for binding with fluorescently-tagged JB6 1-110-CtermCys. Despite large variations, an upward trend in anisotropy was evident at SBDβ concentrations below 20 µM (figure 4). Above 20 µM, the increase in anisotropy diminished, with a slight decrease observed over 100 µM SBDβ. Fitting of these preliminary data suggested a Kd of 1~2 µM.

These FPA results are initial validation of the auto-inhibition release hypothesis. The small molecular weight increase upon binding compared to JB6 1- 110 alone results in only slight reduction in tumbling rate, and thus low signal observed in the FPA assays. The decrease in anisotropy above 100 µM may indicate SBD β self-aggregation via their client- binding cleft8, and compete against JB6 binding. The Kd observed here with JB6 1-110 is consistent with previous FPA analysis of SBDβ and Hsc70 in the ADP state with the client-mimetic NR peptide8, suggesting the G/F-linker can be recognized and bound by the chaperone as a client.

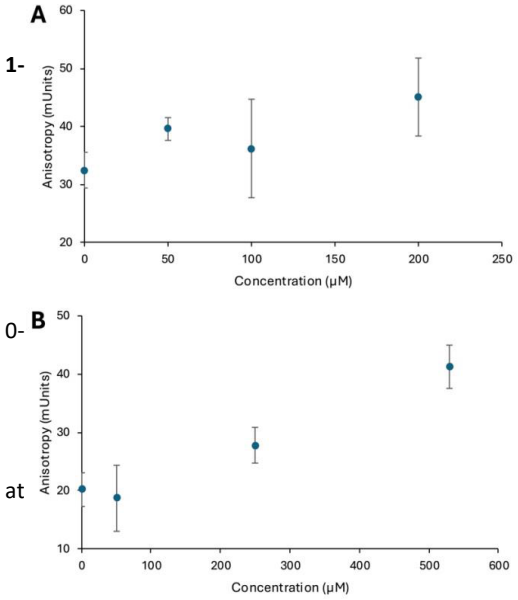

Figure 3. JB6 110 binding to Hsc70 was inhibited. A. FPA data showed significant variations at concentration range of 200 µM. **B.** A more significant upward trend was observed higher Hsc70 concentration.

Future work

The FPA experiment conducted here can be applied to further study the interaction between DNAJB6 and Hsc70, especially at probing the role of the G/F- linker in mediating this intermolecular interaction. JB6 1-69-CtermCys, containing only the J-domain, will function

as a positive control for Hsc70 binding and as negative control for SBDβ interaction1–3. The 1-97-CtermCys construct, which includes the J- domain and G/F-linker before the auto-inhibitory helix, is expected to show higher Hsc70 affinity and similar SBDβ binding as 1-110. Direct competition assays with client-like peptides to displace JB6 from SBD β will provide initial validation for the auto- inhibition release hypothesis.

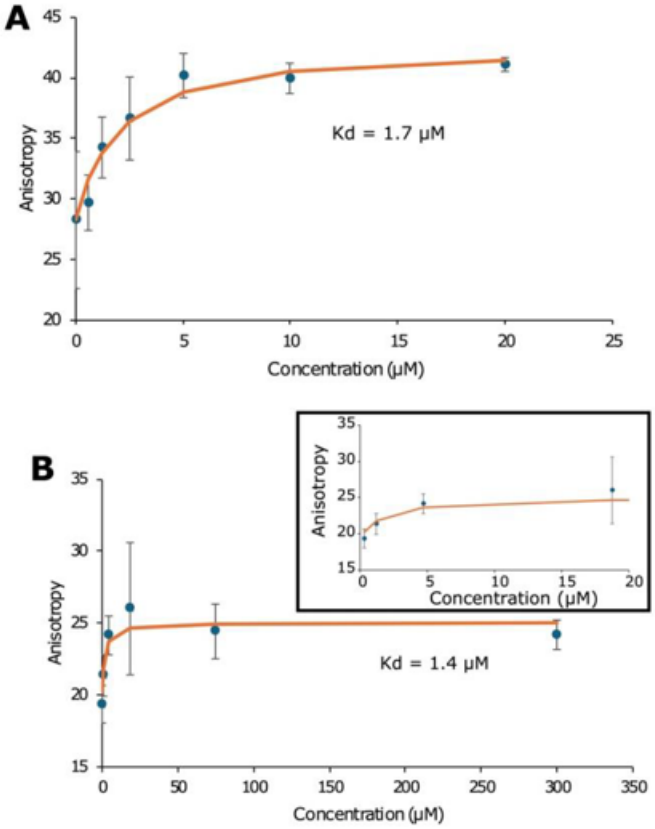

Figure 4. SBDβ showed low affinity binding to JB6 1-110. A. At SBDβ below 20 μM, an upward trend in anisotropy was observed. **B.** Anisotropy showed decrease at SBD β concentration over 100 μM. Inset shows enlarged view of the region of 0-20 SBDβ. Orange line indicates fitted curve.

Impacts & Skills

In this internship, biophysical analysis was conducted to characterize the intermolecular interactions involving class B G/F-linker. The observed binding with Hsc70 SBDβ demonstrates the feasibility of the proposed auto-inhibition release mechanism, and is the first step to understand the intricate balance of the need for Hsc70 activation and the risk of its depletion in living cells. This could contribute directly to our understanding of the molecular mechanism of many neurodegenerative and neuromuscular diseases like limb-girdle muscular dystrophy type-1D.

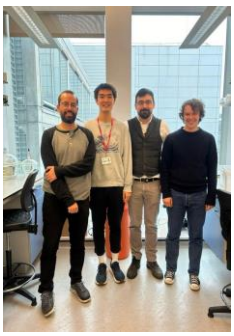

Figure 5. Karamanos lab. From left: Dr. Theodoros Karamanos; Qianyi Zhang; Dr. Felipe Ossa; Billy Hobbs.

This internship is also invaluable for my professional development. I gained essential lab experience, particularly in protein purification via common chromatography methods, a key step in structural characterization. I also developed transferable skills, such as applying constructive feedback in experimental design, data analysis and presentation, while improving collaboration skills with peers and superiors. Additionally, this funding application experience exercised my ability to effectively communicate research to both scientific and general audiences. These experiences will directly prepare me for the Master's program and future progression into an independent researcher.

Acknowledgements

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Reference

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