Characterization of disaggregase activity of a novel chaperone protein

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Abstract

Proteins need to be folded correctly to become functional for the cell, otherwise they can form aggregates. Chaperones are proteins that help with protein folding. A subset of chaperones known as disaggregases can resolve aggregates to their unfolded state. Hsp104 is a disaggregase type chaperone that can unfold disordered as well as ordered aggregates like amyloids. In addition to disaggregating amyloids, Hsp104 can cleave amyloids into smaller propagons which are required for seeding new amyloids and propagating them to daughter cells. Though well conserved in other kingdoms of life, Hsp104 is not present in animals.

In this research, we focus on Abcf2, a new disaggregase candidate, encoded by *S. cerevisiae* gene *ARB1*. This chaperone is present in all animal genomes. To complement *in vivo* assays of disaggregase activity, we conducted *in vitro* assays including semi-denaturing agarose gel electrophoresis (SDD-AGE) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analyses to determine the extent of its activity on Htt-Q103 amyloid, which is derived from the human Huntingtin gene.

What is a disaggregase?

Hsp104 is a concentration-dependent disaggregase

Hsp104 functions differently towards amyloids depending on concentration.

- Low concentration: promotes formation of amyloids
- High concentration: prevents new amyloid formation

Figure 1: mechanism of Hsp104 in amyloid formation.

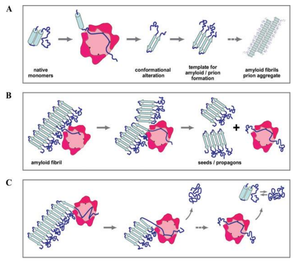


Figure 1: mechanism of Hsp104 in amyloid formation. (A) Hsp104 interacts with native protein to make them amyloid-priming structures. (B) Hsp104 splits up fibrils to make amyloid seeds, thus accelerating amyloid formation. (C) Activity of Hsp104 may lead to complete dissociation of the fibril. (Grimminger-Marquardt & Lashuel, 2010)

Figure 2: the dependence of concentration of Hsp104 activity

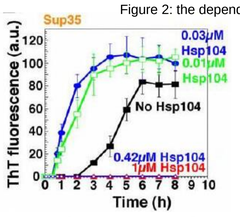


Figure 2: Thioflavin-T fluorescence shows the amount of Sup35 amyloids formed over time. While amyloids formed faster for treatments with low concentrations, no Sup35 amyloid was present with high concentrations of Hsp104 (Shorter & Lindquist, 2006).

New1 is another disaggregase independent of Hsp104

Figure 3: The Disaggregase Ability of New1.

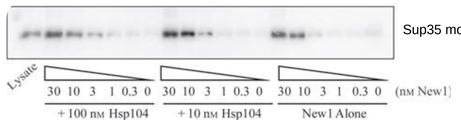
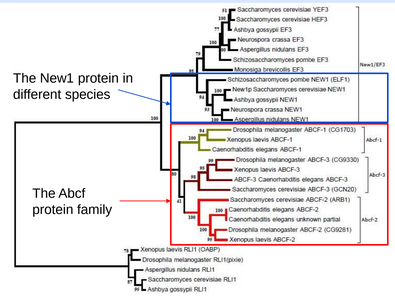


Fig. 3: Bead-tethered Sup35NMHA (amyloid) fibrils were incubated with wild-type yeast lysate (leftmost lane) or with various concentrations of New1 and Hsp104 for 1 h at 30 degrees Celsius. The released Sup35NMHA was detected by Western blotting (Inoue *et al.*, 2011). The amount of monomer increases following the increase of New1, but does not change when the concentration of Hsp104 changes.

The Abcf protein family is closely related to New1

We found that the Abcf protein family is similar to the New1 protein (Fig. 4). Therefore, we hypothesized that the Abcf proteins, including Abcf-2 (Arb1 in *S. cerevisiae*, the focus of our research), have disaggregase activity similar to New1.

Figure 4: a phylogenetic tree showing the relationship between New1 and the Abcf protein family.



Arb1 contributes to Htt-Q103-GFP aggregate processing

We used different strains of cells in *in vivo* assays, including wild type strains (no deletions), *HSP104* deletion strains (*Δhsp104*), and *NEW1* deletion strains (*Δnew1*). Since the *ARB1* gene is essential for yeast viability, we could not use a deletion. Instead, we used the *ARB1* depletion construct consisting of a UAS_{GAL} enhancer. The UAS_{GAL}-*ARB1* strain has normal level of Arb1 when grown on galactose, but Arb1 expression is reduced when glucose is used to grow cells.

Strains with various genotypes including Arb1 depletion, were transformed with a plasmid expressing the amyloidogenic Htt-Q103-GFP protein. Aggregates were detected as GFP positive puncta using fluorescence microscopy. The number of cells with aggregates was counted in several fields.

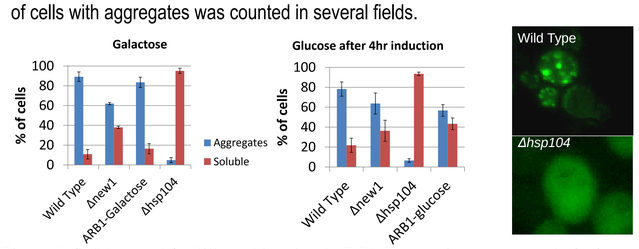


Figure 5 (left 2 panels): Different levels of ARB1 expression were created when strains grow under galactose or glucose conditions. Using fluorescence microscopy, aggregates were detected as GFP-positive puncta. The number of cells with aggregates were counted in several fields (Skuodas and Fassler, unpublished). Figure 6 (right): cells with puncta suggests there are amyloids present (top), while puncta are absent from cells without aggregates (bottom) (Skuodas and Fassler, unpublished).

Figure 5 shows that Arb1 has disaggregase activity as strains with reduced Arb1 levels (right) have fewer GFP puncta. The reduction in the number of amyloids is a characteristic of the *Δhsp104* strain suggesting that Arb1 has a relatively low level of Hsp104-like activity.

In vitro assays are necessary for quantifying the activity of Arb1 on amyloids

Although the *in vivo* assays suggest that Arb1 has disaggregase activity, they do not distinguish between cells with one aggregate and cells with many aggregates. To determine the effect of Arb1 on the total amount of aggregates, we conduct *in vitro* assays using gel electrophoresis of protein extracts to separate amyloids from monomeric proteins, which will provide information about the total amount of aggregated protein associated with each genotype.

Methods

SDD-AGE is the main *In vitro* approach to detect Arb1 activity on Htt-Q103-GFP

- Semi-denaturing detergent-agarose gel electrophoresis
- Agarose provides big enough pores for aggregates
- Separate protein in different form: aggregates, oligomers, monomers
- Electric field: difference of distance depending on size of proteins

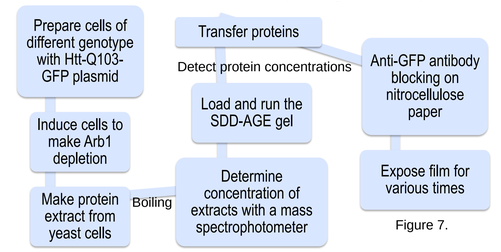


Figure 7.

Figure 7 shows how we processed the SDD-AGE analysis for yeast protein extracts. While we expected a large amount of amyloids in the wild type strain and a large amount of monomeric proteins in the Δhsp104 strain, intermediate amounts of aggregates in Δarb1 and Δnew1 strains were expected as well (Fig. 9).

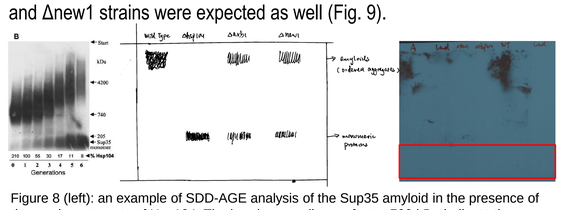


Figure 8 (left): an example of SDD-AGE analysis of the Sup35 amyloid in the presence of decreasing amounts of Hsp104. The bands spreading up from ~700 kDa indicate the presence of protein aggregates, while the lower bands show the monomers. (Kryndushkin et al., 2003). These results show that Hsp104 is necessary to fragment amyloid polymers.
Figure 9 (middle): the expectation of each genotype under SDD-AGE analysis based on the results of *in vivo* analysis conducted before.
Figure 10 (right): SDD-AGE analysis to determine the disaggregase activity of Arb1. Marked from left to right is Δarb1, Δnew1, Δhsp104, and Wild Type strain respectively. There are no monomers present in the red rectangle though it is expected for some strains.

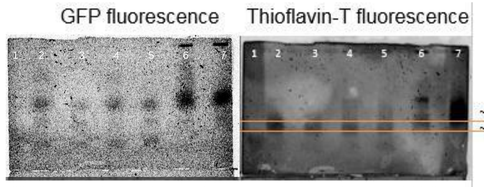


Figure 12: different methods detecting proteins on an SDD-AGE gel: GFP and thioflavin-T fluorescence before the transfer, Ponceau-S stain to confirm transfer, and GFP antibody binding.

- Boiled samples have smaller aggregates
- Top/Bottom band on GFP fluorescence
- Thioflavin-T shows monomers rather than aggregates

SDS-PAGE for detection of monomers continually absent from SDD-AGE

We noticed that monomers are absent in our SDD-AGE analyses. To confirm the existence of monomers expected to be present in Δ*hsp104*, Δ*arb1* and Δ*new1* strains and to identify the aggregates which would appear near the wells of the gel, we conducted an SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) assay (Fig. 10), which separates protein monomers by its molecular weight as SDS denatures proteins. SDS-PAGE helps us better compare monomeric protein levels for different strains.

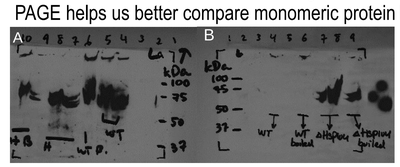
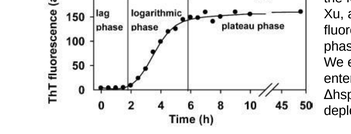


Figure 10 (Left): SDS-PAGE analysis conducted with extracts made from different dates (July 18, 2017 and July 20, 2017 respectively; exposure time ~30 minutes) confirmed the presence of monomers at the size of ~65kDa in the protein extracts, but some unexpected results are still present – the wild type strain (Lane 4 and 5 in Panel A) should not have monomers, and the boiled wild type strain (Lane 6 in Panel B) should have monomers instead of aggregates. These abnormalities may be the result of mistreatment of protein extracts (e.g. mistimed boiling or freezing).

Figure 11 (down): SDS-PAGE analysis for different protein extracts. A4, A10, and B6 should show more proteins, while A6 should show fewer. Reasons for unexpected results may include mistreatment of transfer, boiling, or induction.

Figure 11: SDS-PAGE analysis on all extracts



Gel A	Gel B
4 – *ARB1*-Glucose	4 – Wild Type
6 – *ARB1*-Galactose	6 – *Δnew1* (boiled)
8 – *Δnew1*	8 – *ΔHsp104* (boiled)
10 – *ΔHsp104*	10 – Wild Type (boiled)

Thioflavin-T can track the formation of amyloids

	Thio-T	GFP
Htt-Q103 Monomers		Yes
Htt-Q103 Amyloids	Yes	Yes
Other Amyloids	Yes	

We as well wanted to ensure that no transferred amyloids are absent because they do not bind to α-GFP antibody. Thioflavin-T is a useful tool for tracing the formation of amyloids (Biancalana & Koide, 2010). It binds to amyloids (both Htt-Q103 and other amyloids) and emits fluorescence after it binds with them.

Conclusion

We attempted to use a variety of methods to characterize the disaggregase activity of Arb1 on amyloids. Through this process with some unexpected results present, we analyzed the problems and gained experience of treating protein extracts using different methods and obtained knowledge about the advantages and concerns of each method. We can also use our results to quantify the disaggregase activity of Arb1.

We believe that these experiences on the assays can help further research not only on our research of Arb1, but on chaperones with disaggregase activity as well. The identification of disaggregases can assist on the tackling of amyloid-linked diseases, such as the Huntington's Disease, which is caused by the Htt-Q103 amyloid used in our assays.

Future directions

- Evaluate the viability of using GFP fluorescence instead of antibody treatment to detect monomers of Htt-Q103-GFP
- Use different concentrations of Thioflavin-T on SDD-AGE
- Conduct *in vitro* assays characterizing the disaggregase activity of Arb1 on disordered aggregates
- Purify Arb1 to test its ability to disaggregate amyloids *in vitro*, using Thioflavin-T fluorescence to monitor amyloid formation over time (Fig. 13)

Figure 13: an example of thioflavin-T tracing the formation of amyloids over time (Zhang, Xu, and Perrett, 2010). The level of fluorescence often follows three phases: lag phase, logarithmic phase, and plateau phase. We expect that the wild type strain would enter the log phase with shortest time, the Δhsp104 strain with the longest, and the Arb1 depletion strain located in the middle.

References

Grimminger-Marquardt, V., & Lashuel, H. A. (2010). Structure and function of the molecular chaperone Hsp104 from yeast. *Biopolymers*,*93*(3), 252-276.
Glover, J. R., & Lindquist, S. (1998). Hsp104, Hsp70, and Hsp40: A novel chaperone system that rescues previously aggregated proteins. *Cell*, *94*(1), 73-82.
Shorter, J., & Lindquist, S. (2006). Destruction or potentiation of different prions catalyzed by similar Hsp104 remodeling activities. *Molecular Cell*, *23*(3), 425–438.
Biancalana, M., & Koide, S. (2010). Molecular mechanism of Thioflavin-T binding to amyloid fibrils. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1804*(7), 1405-1412.
Kryndushkin, D. S., Alexandrov, I. M., Ter-Avanesyan, M. D., & Kushnirov, V. V. (2003). Yeast [PSI+] Prion Aggregates Are Formed by Small Sup35 Polymers Fragmented by Hsp104. *Journal of Biological Chemistry, 278*(49), 49636-49643.
Zhang, H., Xu, L., & Perrett, S. (2011). Studying the effects of chaperones on amyloid fibril formation. *Methods*, *53*(3), 285-294.
Inoue, Y., Kawai-Noma, S., Koike-Takeshita, A., Taguchi, H., & Yoshida, M. (2011). Yeast prion protein New1 can break Sup35 amyloid fibrils into fragments in an ATP-dependent manner. *Genes to Cells*, *16*(5), 545-556.