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Reversing deleterious protein aggregation with re-engineered protein disaggregases

Meredith E Jackrel and James Shorter*

Department of Biochemistry and Biophysics; Perelman School of Medicine at the University of Pennsylvania; Philadelphia, PA USA

Aberrant protein folding is severely problematic and manifests in numerous disorders, including amyotrophic lateral sclerosis (ALS), Parkinson disease (PD), Huntington disease (HD), and Alzheimer disease (AD). Patients with each of these disorders are characterized by the accumulation of mislocalized protein deposits. Treatments for these disorders remain palliative, and no available therapeutics eliminate the underlying toxic conformers. An intriguing approach to reverse deleterious protein misfolding is to upregulate chaperones to restore proteostasis. We recently reported our work to re-engineer a prion disaggregase from yeast, Hsp104, to reverse protein misfolding implicated in human disease. These potentiated Hsp104 variants suppress TDP-43, FUS, and α-synuclein toxicity in yeast, eliminate aggregates, reverse cellular mislocalization, and suppress dopaminergic neurodegeneration in an animal model of PD. Here, we discuss this work and its context, as well as approaches for further developing potentiated Hsp104 variants for application in reversing protein-misfolding disorders.

Keywords: Hsp104, protein-misfolding disorders, proteostasis, amyloid, disaggregation, neurodegeneration

Abbreviations: Hsp, heat shock protein; ALS, amyotrophic lateral sclerosis; PD, Parkinson disease; HD, Huntington disease; AD, Alzheimer disease; α-syn, α-synuclein; NBD, nucleotide binding domain; AAA+, ATPases associated with diverse activities

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*Correspondence to: James Shorter;
Email: jshorter@mail.med.upenn.edu

Proteins must fold properly to function effectively.[1] Therefore, protein folding is essential for all life.[1] Accordingly, protein misfolding is associated with numerous fatal neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS), Parkinson disease (PD), Huntington disease (HD), and Alzheimer disease (AD).[1-3] Each of these disorders is associated with the accumulation of misfolded aggregates of specific proteins.[2] In certain patients with ALS, the RNA-binding proteins TDP-43 and FUS form aggregates that accumulate in the cytoplasm, whereas these proteins would normally be predominantly soluble within the nucleus and shuttling to and from the cytoplasm.[4-8] In patients with PD, α-synuclein (α-syn) takes on a cross-β amyloid conformation and accumulates in Lewy bodies.[9] While amyloid accumulation has long been presumed to be the underlying cause of α-syn toxicity, and, indeed, pure amyloid forms of α-syn suffice to initiate disease de novo,[10,11] it has also been suggested that it is not the amyloid fibers, but instead the pre-amyloid oligomers that are the toxic species.[1,3] There are no therapies that eliminate any of these misfolded conformers, and available therapeutics remain palliative. It is therefore essential to develop novel strategies to target the underlying cause of these disorders: protein misfolding and aggregation.

Numerous strategies have been explored to reverse protein aggregation and restore proteostasis. One strategy is to enhance the activity of cellular chaperones. For instance, Hsp70 mediates protein folding and serves to proofread and repair proteins.[12] Thus, Hsp70 is a plausible candidate for upregulation to enhance protein folding and restore proteostasis. Indeed, upregulation of Hsp70 suppresses polyglutamine-induced neurodegeneration in *Drosophila*.[13,14] Moreover, Hsp70 is a critical component of the protein disaggregase machinery of metazoa, and when combined with Hsp110 and Hsp40 can resolve disordered aggregates and slowly depolymerize amyloid.[15-19] Chaperones in the Hsp100 family of proteins possess an even more powerful and

rapid disaggregase activity, disassembling proteins from aggregated conformations in collaboration with Hsp110, Hsp70, and Hsp40.[16,17,20,21] Using proteins in the Hsp100 family to reverse human disease phenotypes is intriguing, because the ability of Hsp100 proteins to rapidly untangle proteins from pre-existing inclusions would seem to have far greater therapeutic value than solely preventing aggregation.[20,22] While Hsp100 proteins are highly conserved in eubacteria and the vast majority of eukaryotes, Hsp104 from the budding yeast *Saccharomyces cerevisiae* is of particular interest, because it is the only protein known to rapidly disassemble amyloid and toxic preamyloid oligomers, but has no metazoan homolog.[20,22-24] *S. cerevisiae* maintain a complex mechanism of genetic regulation requiring the construction and disassembly of yeast prions, and Hsp104 is critical in mediating these processes.[25] Our lab and others have explored applying Hsp104 to human disease models.[14,23,24,26,27] In addition to applying native Hsp104, we have also aimed to engineer Hsp104 to have enhanced activity against different substrates implicated in neurodegeneration in an effort to restore proteostasis.[26] Recently, we reported the development of potentiated Hsp104 variants that disaggregate TDP-43, FUS, and α-syn.[26] The potentiated Hsp104 variants not only suppress toxicity of these substrates in yeast, but they also restore the proper localization of these proteins and mitigate neurodegeneration in a *C. elegans* model of PD. Here, we further discuss the context of our work, the isolation of these variants, as well as future directions in the development of Hsp104 variants for use in a therapeutic context.

Applying Chaperones to Restore Proteostasis

Overexpression of expanded polyglutamine tracts causes neurodegeneration in *Drosophila*, providing a useful platform to assess the reversal of polyglutamine-mediated neurodegeneration.[13] Using this model, Hsp70 was discovered to suppress aggregation of polyglutamine and degeneration in *Drosophila*.[14,28] Additionally, co-expression of human Hsp110 and Hsp40 suppresses the toxicity and aggregation of polyglutamine in *Drosophila*.[29] We recently expressed Hsp70 or Hsp104 in a *Drosophila* model of spinocerebellar ataxia.[14] Here, Hsp104 suppressed toxicity after the onset of polyglutamine-induced degeneration.[14] Importantly, in the same system, while Hsp70 inhibits degeneration, it is unable to reverse degeneration.[14] Hsp104 has also been demonstrated to eliminate aggregates of various other substrates implicated in neurodegenerative disease.[23,24] Hsp104 disassembles preformed α-syn amyloid fibers as well as its preamyloid oligomeric precursors.[23,24] In addition, Hsp104 suppressed α-syn-induced dopaminergic neurodegeneration in the rat substantia nigra.[24] Furthermore, we have demonstrated that Hsp104 disassembles preformed aggregates and amyloids comprised of Aβ, tau, polyglutamine, and amylin in vitro.[23] However, these activities were modest and required very high levels of Hsp104, which presents a barrier to further developing Hsp104 as a strategy for reversing neurodegeneration. We recently broached this issue with the development of re-engineered Hsp104 variants.[26] These potentiated variants are highly active, with disaggregase and ATPase activity far greater than wild-type Hsp104 ($Hsp104^{WT}$). Additionally, in yeast and *C. elegans*, these variants suppress toxicity, reverse mislocalization, and suppress dopaminergic neurodegeneration in situations where $Hsp104^{WT}$ is ineffective.[26] To isolate these variants, we constructed large libraries of Hsp104 mutants and implemented yeast models of proteotoxicity as a platform to screen these libraries for Hsp104 variants that potently suppress proteotoxicity.

Strategies for Re-Engineering Hsp104

Hsp104 is an AAA+ protein comprised of 5 domains: an N-terminal domain, 2 nucleotide binding domains (NBDs), a middle domain, and C-terminal domain.[30] The protein forms a 2-tiered hexamer with a central pore, through which substrate is threaded.[30,31] Hsp104 is very large, consisting of 6 102-kDa monomers, and its structure remains poorly understood. Therefore, Hsp104 is a relatively poor candidate for rational design.[32] Initially, we hypothesized that the substrate-binding loop regions that line the central channel of Hsp104[31] could be optimized against specific substrates. However, we have noted only subtle rescue phenotypes and more typically detrimental effects when mutating these positions.[26] Therefore, we instead adopted a broader approach to library construction. We utilized a domain-specific random mutagenesis approach to construct libraries with high diversity in restricted regions of the protein. We selected the middle domain of Hsp104 for randomization, because the middle domain facilitates ATPase activity, communication between NBD1 and NBD2, intrinsic disaggregase activity, and interactions with Hsp70.[26,30] Restricting mutations to just the middle domain was key, as limiting the size of the randomized region allowed for greater depth of coverage. Additionally, this strategy allowed for sequencing to be restricted to a length allowing a single sequencing reaction per variant.

Yeast Models of Proteotoxicity

Yeast are an ideal model system for studying protein misfolding, as they grow rapidly, and excellent genetic tools are available to manipulate yeast.[33] Additionally, the yeast genome is highly conserved to that of humans. Thus many fundamental cellular processes, including mechanisms for the regulation of proteostasis, are highly conserved between yeast and mammalian cells.[33,34] Furthermore, upon overexpression of several different substrates implicated in various neurodegenerative diseases, many phenotypes noted in patients with these disorders are recapitulated in yeast.[33,34] For instance when TDP-43 and FUS are overexpressed in yeast, they form cytoplasmic aggregates, while normally the proteins should translocate between the nucleus and cytoplasm.[35-37] Similarly, in certain patients with ALS, cytoplasmic aggregates of TDP-43 or FUS are present, and the

proteins are thus excluded from entering the nucleus.[4] When α-syn is overexpressed in yeast, it also forms cytoplasmic aggregates.[38] In PD patients, cytoplasmic aggregates of α-syn are found, while normally the protein should accumulate at the plasma membrane.[9] Thus, yeast provide an excellent platform for screening proteins or other agents for toxicity suppressors. Indeed, yeast overexpressing TDP-43 was used to identify Pbp1 as an enhancer of TDP-43 toxicity.[39] It was then determined that polyglutamine expansions in the human homolog of Pbp1, ataxin 2, is one of the leading genetic risk factors for ALS.[39] Additionally, yeast overexpressing α-syn was used to screen small molecules to identify those that protect cells from α-syn aggregation and toxicity.[40] Using these methods, the compound NAB2 was identified to rescue α-syn pathological phenotypes in cortical neurons from iPS cells of PD patients harboring α-syn mutations.[40,41] Thus, we envisioned these yeast models to be an ideal platform for screening libraries of Hsp104 variants.

Isolating Potentiated Hsp104 Variants

Using these yeast proteinopathy models, we screened the library of Hsp104 middle domain variants and isolated a series of potentiated variants.[26] We identified point mutations in helices 1, 2, and 3 of the middle domain, as well as in the small domain of NBD1.[26] At several of these positions, we introduced additional mutations to other amino acids.[26] Surprisingly, at each position we tested, we could introduce both conservative and nonconservative mutations to potentiate Hsp104. We selected alanine 503 to mutate to all amino acids, and, astonishingly, we could mutate position 503 to any amino acid, with the exception of proline, to activate Hsp104.[26] There are no other examples, to our knowledge, of instances where mutation to any amino acid yields the same gain of therapeutic function phenotype. We hypothesize that loss of amino acid identity at this position and select others likely leads to a structural perturbation that mimics an allosteric activation event.

Hsp104 has likely been tuned through evolution to be highly regulated in recognizing substrates appropriate for disaggregation. Dysregulation of Hsp104 substrate recognition could lead to unfolding of essential proteins and, therefore, toxicity. Thus, it is very surprising that such a diverse array of mutations in disparate regions of the protein can lead to a generic gain of therapeutic function. Therefore, we suggest that Hsp104 has been tuned to be a tightly regulated protein. We propose that the middle domain serves as a capacitor, which is braced to unleash activity. Fine structural changes in the middle domain perturb the structure sufficiently to activate Hsp104, leading to unfolding of nearly any misfolded substrate encountered. This dysregulation is likely the source of the subtle temperature-sensitive phenotype at elevated temperatures (poor growth at 37 °C) noted for some of the variants.[26]

Testing Hsp104 Variants in Animal Models

While yeast are an ideal model system for screening for potentiated Hsp104 variants and assessing aggregation, they can never fully recapitulate the complex biology of the metazoan nervous system. Thus, it is essential to also assess candidates that reverse protein aggregation in animal models. We tested a subset of the variants in a transgenic *C. elegans* model of PD. *C. elegans* are an ideal system for studying neurodegeneration, because they are of invariant lineage, and the dopaminergic neurons can be easily counted by co-expressing a fluorescent marker protein targeted selectively to the neurons. α-Syn can be targeted to the dopaminergic neurons, leading to a time-dependent loss of neuronal activity.[26,42,43] We targeted α-syn and Hsp104 to the dopaminergic neurons, and observed for $Hsp104^{A503S}$ and $Hsp104^{Y257F:A503V:Y662F}$ a strong inhibition of dopaminergic neurodegeneration.[26] It will next be essential to test these variants in mammalian models, such as the rat substantia nigra model used to establish suppression of α-syn toxicity and neurodegeneration by $Hsp104^{WT}$.[24]

Fine-Tuning Potentiated Hsp104 Activity

Potentiated Hsp104 variants are highly promising for reversing protein aggregation disorders. To our knowledge, there are no other molecules known, protein or otherwise, that reverse TDP-43, FUS, and α-syn aggregation. However, the variants that we have developed are non-specific. That is, most of the potentiated Hsp104 variants suppress the toxicity of TDP-43, FUS, and α-syn. While none of the variants are specific to a single substrate, $Hsp104^{D498V}$, $Hsp104^{Y507V}$, and $Hsp104^{D504C}$ strongly rescue α-syn and FUS toxicity, but not TDP-43 toxicity.[26] $Hsp104^{D504V}$ behaves similarly, strongly rescuing α-syn and FUS toxicity but only mildly rescuing TDP-43 toxicity.[26] Thus, these variants are likely activated against many other substrates that may not necessarily be misfolded. We hypothesize that this non-specific activity is the basis for the temperature-sensitive phenotype (poor growth at 37 °C) we have noted for several variants, which ranged from subtle for $Hsp104^{A503S}$ and $Hsp104^{Y257F:A503V:Y662F}$ to more severe for $Hsp104^{A503V}$ [26]. However, the variants do not display a growth defect at 30 °C, so we do not believe this to be a problematic phenotype.[26] Nonetheless, to move forward in applying Hsp104 variants to different diseases, it will be essential to fine-tune their substrate specificity. For instance, variants that are selected for disaggregation of TDP-43 but do not suppress α-syn toxicity will likely have greater overall substrate specificity and a diminished temperature-sensitive phenotype.

Using the methods we have developed, it will be challenging to identify variants that disaggregate a single substrate, as this could potentially entail manual screening of many variants. Thus, it will be important to develop new strategies for identifying substrate-specific variants. Additionally, it will be important to implement more directed approaches to variant design to develop substrate-specific variants. For instance, mutating the substrate-binding tyrosine residues that line the pore of Hsp104 in a potentiated background may allow for enhanced substrate specificity.

Outlook

The application of chaperones to reversing neurodegenerative disease is a promising approach for restoring proteostasis. Hsp104 is a particularly promising chaperone for use in this context, as it is uniquely capable of rapidly disassembling amyloid. Alternatively the metazoan Hsp110, Hsp70, and Hsp40 system, which slowly depolymerizes amyloid, could be evolved to enhance its activity.[15] Additionally, Hsp104 could likely be potentiated against any protein, which may be valuable for solubilizing proteins that are difficult to purify.

To further develop Hsp104 variants for potential use as therapeutics, it will be essential to further fine-tune their substrate specificity. It is also essential to further test these variants in rodent models. Ultimately, we envision that direct injection or gene therapy could be used to introduce Hsp104 into patients in transient bursts. Regardless of the potential therapeutic applications of potentiated chaperones, our work[14,26] and that of others[27,28] has established that using chaperones to divert disease-associated proteins to their nonpathogenic states may be a highly promising strategy for halting and reversing neurodegenerative disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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