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Prion-impairing mutations in Hsp70 chaperone Ssa1:

Effects on ATPase and chaperone activities

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Abstract

We previously described many Hsp70 Ssa1p mutants that impair $[PSI^+]$ prion propagation in yeast without affecting cell growth. To determine how the mutations alter Hsp70 we analyzed biochemically the substrate-binding domain (SBD) mutant L483W and the nucleotide-binding domain (NBD) mutants A17V and R34K. Ssa1^{L483W} ATPase activity was elevated 10-fold and was least stimulated by substrates or Hsp40 co-chaperones. Ssa1^{A17V} and Ssa1^{R34K} ATPase activities were nearly wild type but both showed increased stimulation by substrates. Peptide binding and reactivation of denatured luciferase were enhanced in Ssa1^{A17V} and Ssa1^{R34K} but compromised in Ssa1^{L483W}. The nucleotide exchange factor Fes1 influenced ATPase of wild type Ssa1 and each mutant differently. Partial protease digestion uncovered similar and distinct conformational changes of the substrate-binding domain among the three mutants. Our data suggest that prion-impairing mutations of Ssa1 can increase or decrease substrate interactions, alter the Hsp70 reaction cycle at different points and impair normal NBD-SBD cooperation.

Keywords

Hsp70; prion; yeast; chaperone; $[PSI^+]$

Introduction

Hsp70s are universally conserved and essential protein chaperones that protect proteins during stress and help proteins fold, cross membranes, assemble and disassemble into complexes and become degraded [1-5]. Hsp70s are involved in a large number of human diseases and the mechanisms of Hsp70 function are an important clinical consideration [6].

In the yeast *Saccharomyces cerevisiae* several infectious amyloids (prions) have been described that are transmitted between cells during mating and cell division [7-9]. Prion propagation in yeast is an incompletely understood process that involves a complex network of chaperone activities. Changes in expression levels or function of any of several major chaperones or co-chaperones can influence prion propagation [10-14]. Recently, several studies have investigated the effect of single proteins or small mixtures of proteins on the dynamics of amyloid growth in vitro [15-17]. While these experiments provide insight into interactions of isolated components of the chaperone machinery with amyloid, it remains

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unclear how accurately they reflect how these and other factors influence dynamics of prion propagation in the highly complex environment of the cell.

Altered function of the Ssa cytosolic Hsp70 family considerably impairs propagation of the yeast prion [*PSI*⁺], an infectious amyloid form of the translation termination factor Sup35 [18-21]. The Ssa family consists of four highly conserved proteins. Constitutively expressed Ssa1 and Ssa2 are 98% identical, while stress-inducible Ssa3 and Ssa4 are 88% identical and share 80% identity with Ssa1 and Ssa2. Abundant expression of at least one of the four Ssa proteins is essential for cell viability [22]. Previous mutagenic studies of Ssa1, which is recognized as a critical component of the chaperone machinery involved in prion propagation, have uncovered a large number of single point mutations that weaken and destabilize [*PSI*⁺] [18-20]. Many of these mutations are of additional interest because they have no discernable effect on cell growth.

Hsp70s have a modular structure with adjacent nucleotide binding (NBD) and substrate-binding (SBD) domains. The NBD and SBD functionally interact through an α -helical region of the SBD (the α -A helix) and an inter-domain linker [23]. The original and most characterized mutation L483W is located at the base of the SBD near the NBD. All of the remaining [*PSI*⁺]-altering mutations are in the NBD. Four of them cluster within residues 17-34 located at the base of the NBD near the SBD, suggesting this area is involved in an activity particularly important for [*PSI*⁺] propagation.

Although the effects of the Ssa1 mutations on [*PSI*⁺] are well characterized, how they affect Hsp70 enzymatic activities and functional cycle are only inferred from genetic data. In addition to Ssa1^{L483W}, we chose the Ssa1^{A17V} and Ssa1^{R34K} mutants to examine relationships between mutations in the NBD and SBD, and how the mutations affect enzymatic activity of Ssa1 protein. All three mutations appeared to affect inter-domain communication and substrate interactions but each affected Hsp70 enzymatic activities differently.

Methods

Protein purification

Ssa1 was purified from the yeast strain used for [*PSI*⁺] characterization in our previous genetic screen (*ssa1-4A* strain G400-1C [19] with wild type or mutant Ssa1 expressed from a plasmid). Yeast grown in standard rich medium (YPAD) to an OD₆₀₀ of 10 were collected by centrifugation, suspended in Buffer I (25 mM HEPES pH 7.0, 50 mM NaCl, 10 mM KCl, 5 mM MgCl₂, 2.5% Glycerol, 10 mM β -mercaptoethanol, Protease Inhibitor cocktail [Roche cat# 11836170001]) and broken by vortexing with glass beads. Proteins were loaded on a DEAE column in Buffer I and eluted using a gradient of 50 to 500 mM NaCl. Peak Ssa1 fractions were combined and loaded onto a 5 ml ATP-agarose column (Sigma cat# A2767), washed with 25 ml Buffer I + 500 mM NaCl, then with 25 ml Buffer I. Proteins were eluted with Buffer I + 2 mM ATP, diluted two-fold in 20 mM KPO₄ pH 6.8, and loaded onto an hydroxyapatite column. Proteins were eluted using a gradient of 20 to 300 mM KPO₄ pH 6.8. The elution peak of Ssa and Ssb proteins overlapped somewhat as seen by Cyr [24]. Fractions containing only Ssa1 were pooled, concentrated on Amicon Ultra spin columns (cat# UFC801024), and then applied to a Sephacryl 200 size exclusion column in 20 mM HEPES pH 7.0, 200 mM KCl, 5 mM MgCl₂ to remove small degradation products. Fractions containing full length Ssa1 were concentrated on Amicon Ultra spin columns and then changed into 10 mM HEPES pH 7.0, 50 mM NaCl, 10 mM β -mercaptoethanol, 10% glycerol on a PD-10 desalting column (Amersham cat# 17-0851-01). Ssa1 protein was quick frozen and stored at -80°. No contaminating proteins were detected by SDS-PAGE and Coomassie staining of 5 μ g samples. Typical yields were 2-4 mg Ssa1 protein from 2L of cell culture.

Co-chaperone proteins were purified from *E. coli* BL21 grown in LB medium containing antibiotic appropriate for plasmid maintenance. Ydj1 was purified on DEAE and hydroxyapatite as described previously [25]. Sis1 was purified similarly on sulfopropyl and hydroxyapatite. Fes1 was purified as a GST fusion on glutathione sepharose as described [26]. All purified proteins were changed into 10 mM HEPES pH 7.0, 50 mM NaCl, 10 mM β -mercaptoethanol, 10% glycerol on a PD-10 desalting column (Amersham cat# 17-0851-01) and stored at -80° .

Hsp104 was expressed in *E. coli* BL21 RIL. One liter of culture was grown to OD 0.4 and induced with 0.5 mM IPTG for 1 hr at 37° . Cells were harvested by centrifugation and suspended in 50 mM Tris-Cl pH 7.4, 2 mM EDTA 5 mM $MgCl_2$, 5% Glycerol, 0.1% Triton-X100, 1 mM PMSF, Protease Inhibitor cocktail [Roche cat# 11836170001]. After adding lysozyme to 0.5 mg/ml, cells were incubated on ice for 20 min and sonicated. Proteins were precipitated by slowly adding concentrated PEG8000 dissolved in 20 mM Tris-Cl pH 7.4 with stirring to a final concentration of 15%. Precipitated protein was pelleted by centrifugation, suspended in Buffer I + 5 mM β -mercaptoethanol, applied to DEAE and eluted with a gradient of 50-500 mM NaCl. Pooled fractions were dialyzed two hrs in 20 mM KPO_4 pH 6.8 then applied to hydroxyapatite and eluted with a 20-300 mM KPO_4 gradient. Purified Hsp104 was concentrated on Amicon spin columns, dialyzed into 50 mM Tris-Cl pH 7.4, 5 mM $MgCl_2$, 10% Glycerol, 5 mM β -mercaptoethanol, and stored at -80° .

Protease digestion

Ssa1 protein (10 μ g) was incubated in 25 μ l of 25 mM HEPES pH 7.3, 50 mM KCl, 5 mM $MgCl_2$ with 5 mM ATP or ADP for 20 minutes at 25° . Proteinase K (Invitrogen) at 30 μ g/ μ l was diluted 1/100 in assay buffer and 1 μ l added to each reaction. Aliquots were removed and added to equal volumes of cold 40% TCA, incubated on ice for 30 minutes and then centrifuged at 14000 RPM for 20 minutes. Pellets were washed in cold acetone, dried, and suspended in 1X SDS buffer.

ATPase assays

ATP hydrolysis was measured in 25 mM Tris-Cl pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 1mM DTT in a 200 μ l volume at 25° . In reactions measuring stimulation of ATPase by protein substrates, reduced carboxy-methyl lactalbumin (rCMLA, Sigma cat# L5888) or A7 peptide (Arg-Arg-Leu-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, Sigma cat# A7433) were used. Peptide substrate, Ydj1, Sis1, and GST-Fes1 were added at indicated concentrations to 0.4 μ M Ssa1. Reactions were incubated for 10 min at 25° and the assay started by addition of [γ - ^{32}P] ATP to a final concentration of 50 μ M Aliquots were removed at 10, 20, and 30 minutes and the amount of ATP hydrolysis determined as described [27]. ATP turnover rates were linear over the course of the assay and were highly reproducible over multiple separate protein preparations.

Luciferase refolding

For reactions containing only Ssa1 and Ydj1, recombinant firefly luciferase (Promega cat# E1701) at 80 nM concentration in 25 mM Tris-Cl pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 1mM DTT was denatured by incubating at 42° for 15 min in the presence of 1 μ M Ydj1. Denatured luciferase was diluted 1/2 into a 50 μ l refolding reaction with final concentrations of 0.8 μ M Ssa1, 1.5 μ M Ydj1, 5 mM ATP. For reactions containing Hsp104, luciferase was denatured in 6M Urea, diluted into ice cold refolding buffer, then added to chaperones as described by Glover [28]. Final concentrations were 1 μ M each Hsp104, Ssa1, and Ydj1; 20 nM luciferase, 5 mM ATP, 20 mM phosphocreatine and 0.2 mg creatine phosphokinase. Aliquots were removed at various times and assayed for luciferase activity using a Zylux FB15 luminometer

and luciferase assay reagent (Promega cat# E1501). Spontaneous refolding of luciferase without added chaperones was subtracted as background.

Fluorescence anisotropy

Peptide A7 was dissolved in 100 mM NaPO₄ pH 8.0 at a concentration of 0.5 mg/ml, and 100 μ l was incubated in the dark at 4° overnight with 10 μ l of 2 mg/ml Fluorescein Isothiocyanate in DMSO (Sigma cat# F7250). Labeled peptide (F-A7) was separated from un-reacted dye by gel filtration on a Sephadex G-10 column. F-A7 (100 nM) was incubated with Ssa1 proteins for 1 hr at 32° in 25 mM Tris-Cl pH 7.4, 100 mM KCl, 5 mM MgCl₂, 50 μ M ADP. Fluorescence polarization was measured in a Photon Technology International QM-6/2005 spectrofluorometer at 495 nm excitation and 515 nm emission, and anisotropy values were calculated with Felix 32 software.

Results

Location of mutations

Figure 1 shows the locations of the Ssa1 mutations on the highly conserved structure of *E. coli* Hsp70, DnaK. The structures of the NBD and SBD were determined independently [29, 30] and are oriented on the basis of recent data of a partially intact structure of bovine Hsc70 [23]. ATP binding and hydrolysis drives the functional cycle of Hsp70. ATP-bound Hsp70 has low affinity for substrate and high rates of substrate exchange. ATP hydrolysis controls trapping of peptide substrate by inducing a conformational change in the SBD [31,32]. Interaction of peptide substrate in the binding pocket of the SBD likewise transmits a signal to the NBD that stimulates ATP hydrolysis, thus enhancing its own binding. Re-binding of ATP restores the low affinity conformation, allowing substrate release and completing the cycle. The three mutated residues are not directly involved in ATP hydrolysis or substrate interaction, but are in positions where they might affect communication of signals between the two domains.

Intrinsic ATPase activities of Ssa1 proteins

The high purity of preparations of purified wild type and mutant Ssa1 proteins is demonstrated in Figure 2A. To look for alterations in the ATPase cycle of the Ssa1 mutants we measured the intrinsic steady state rate of ATP hydrolysis (Figure 2B, Int). Wild type Ssa1 activity (.047/min) was in close agreement with previous reports [33, 34]. The activities of the NBD mutants Ssa1^{A17V} (.037/min) and Ssa1^{R34K} (.044/min) were very similar to wild type. Thus the NBD mutations do not seem to affect the ATPase of Ssa1 greatly, although the 20% lower activity of Ssa1^{A17V} was consistently reproducible. The SBD mutant Ssa1^{L483W} had intrinsic ATPase activity (.44/min) nearly 10 fold higher than wild type, suggesting that the L483W substitution causes an inappropriate stimulatory signal to the NBD.

Stimulation of ATPase by peptide substrates is elevated for NBD mutants and reduced for the SBD mutant

We tested stimulation of ATPase by two often-used Ssa1 substrates, the unfolded protein rCMLA and the hydrophobic peptide A7 (see Methods). Consistent with work by others [35], wild type Ssa1 ATPase was moderately stimulated by each substrate (Figure 2B). ATPase stimulated by rCMLA was approximately 50% higher than intrinsic activity. The same concentration of A7 peptide stimulated slightly better (70%) than rCMLA. Doubling the concentration of rCMLA resulted in additional stimulation to nearly 2-fold over the unstimulated activity. This concentration dependence suggests that stimulation of ATPase is closely related to substrate affinity, a correlation seen in other Hsp70s [36].

The ATPase of Ssa1^{A17V} and Ssa1^{R34K} was stimulated 3 to 4 fold by A7 peptide, which was significantly greater than for wild type Ssa1. Differences in ATPase of this magnitude noticeably affect Hsp70 function [23]. Thus while the absolute rate of ATP turnover remains low, the increased ability to be stimulated suggests these mutants more readily transition into the higher affinity peptide-bound state upon interaction with substrate. Unlike wild type, doubling the amount of rCMLA resulted in only a small amount of additional stimulation, suggesting that substrate binding for the NBD mutants may already be near saturation under these conditions. A7 peptide and rCMLA stimulated the high basal ATPase of Ssa1^{L483W} protein by only 20% and 30% respectively, which was less than half that seen for wild-type. Thus, although substrate-stimulated ATPase activity of all three mutants was enhanced, [PSI⁺]-weakening mutations in the NBD and the SBD have different effects on the ATPase cycle of Ssa1.

Substrate binding is elevated for NBD mutants and reduced for the SBD mutant

To investigate substrate interactions further, we measured equilibrium binding of fluorescein-labeled A7 peptide. Binding was done in the presence of ADP to obtain stable protein/peptide complexes [37]. The increase in fluorescence anisotropy over peptide alone, reflecting the binding to Ssa1, is shown in Figure 2C. As expected, for all four Ssa1 proteins the amount of change in anisotropy was dependent on Hsp70 concentration. Ssa1^{A17V} and Ssa1^{R34K} bound more peptide than wild type, while Ssa1^{L483W} bound less. The relative amounts of peptide binding correlate with the relative ability of A7 to stimulate ATPase, which supports the view that the magnitude of ATPase stimulation is at least partially due to substrate affinity. However, since NBD mutants bound the most peptide, the possibility remains that the mutations alter ability of substrate to trigger ATP hydrolysis, and mechanisms other than simple substrate affinity could be involved in the overall effect of the mutations on substrate interactions.

Stimulation of ATPase by Hsp40 is reduced by the SBD mutation and only slightly affected by the NBD mutations

Stimulation of ATP hydrolysis by Hsp40 family co-chaperones is a universal mechanism of regulation and specificity of Hsp70 function [38-40]. We tested relative stimulation of ATPase by the two major yeast Hsp40 co-chaperones Ydj1 and Sis1. Wild type Ssa1 ATPase was stimulated 7 and 5 fold by Ydj1 and Sis1, respectively (Figure 3A). Consistent with previous work Ydj1 stimulated better than Sis1 [33]. The stimulation of Ssa1^{A17V} and Ssa1^{R34K} by Ydj1 and Sis1 varied slightly but the magnitude of the effect was similar to wild type. The SBD mutant Ssa1^{L483W} was stimulated only 2 fold by either Ydj1 or Sis1, suggesting this mutant may be less efficiently regulated by Hsp40s. A recent study that modeled Hsp70 activity based on an extensive matrix of kinetic parameters predicted that Hsp70 with a hyperactive ATPase would be less dependent on co-chaperones [41]. Our results are consistent with such an interpretation for the L483W protein.

The standard assay in Figure 3A used a 4-fold molar excess of Hsp40 to determine ATPase stimulation near maximal levels. To determine if the mutant Ssa1 proteins had altered sensitivity to stimulation at lower Ydj1 concentrations, ATPase was measured with Ydj1/Ssa1 ratios ranging from 0.25 to 4 (Figure 3B). A similar gradual increase in ATPase was seen with increasing Ydj1 concentration for all of the Ssa1 proteins. Although stimulation of ATPase activity for Ssa1^{L483W} was much lower overall, the increase in stimulation showed the same Ydj1 concentration dependence as the other Ssa1 proteins. Thus, none of the mutant proteins had an altered sensitivity to stimulation at lower Ydj1/Ssa1 ratios.

Adding co-chaperone and substrate together stimulates Ssa1 ATPase synergistically [35]. Combining rCMLA with Ydj1 stimulated ATPase of wild type Ssa1 3-fold more than Ydj1 alone (Figure 3C). This co-stimulation was synergistic as rCMLA alone did not stimulate

ATPase by this magnitude (Figure 2). In contrast, combining Sis1 and rCMLA resulted in only a minor increase in ATPase over Sis1 alone. These observations imply that substrate and co-chaperone simultaneously interact with Ssa1 and only certain combinations of Hsp40 and substrate adopt a conformation capable of co-stimulating the ATPase.

The ATPase of Ssa1^{L483W} was increased beyond its high intrinsic activity by the combination of Ydj1 and rCMLA as well as by combined Sis1 and rCMLA. However, the magnitude of this stimulation was small. Thus, the high intrinsic ATPase activity of Ssa1^{L483W} might lessen the influence of co-factors on its functional cycle. The ATPase of Ssa1^{R34K} responded like wild type Ssa1, showing synergistic stimulation by combined Ydj1 and rCMLA, and only a minor increase by combined Sis1 and rCMLA. In contrast, Ydj1 and rCMLA did not synergistically activate ATPase of Ssa1^{A17V}, and the combination of Sis1 and rCMLA actually reduced activity compared with Sis1 alone. Thus, although rCMLA activated both Ssa1^{A17V} and Ssa1^{R34K} much better than wild type, the A17V mutation inhibited co-stimulation by Hsp40 and rCMLA, revealing an important difference between these NBD mutants.

ATPase of each Ssa1 mutant responded differently to the nucleotide exchange factor Fes1

Nucleotide exchange factors (NEFs) are important regulators of the Hsp70 reaction cycle, and even small changes in exchange rates can affect chaperone functions [42-44]. Nucleotide dissociation rates for different Hsp70s can vary by 1000-fold and NEFs are required for proper function of some but not all Hsp70s [45,46]. Thus, the dynamics of nucleotide exchange and the effect on function is specific to each NEF/Hsp70 pair.

We earlier showed that increasing abundance of Fes1, the first NEF identified for Ssa1, improves [*PSI*⁺] propagation in cells expressing Ssa1^{L483W}. Previous reports show Fes1 can enhance or inhibit Ssa1 ATPase *in vitro* [26,47]. These contrasting effects might be explained by a sensitivity of Fes1 to assay conditions. To determine if Fes1 had a positive or negative influence on our proteins, the ATPase activity of each Ssa1 protein in the absence of Fes1 was normalized to 100 and the activity measured when Fes1 was present was compared to this normalized level (Figure 4). Fes1 reduced the intrinsic and stimulated ATPase activities of wild type Ssa1 to about 75%. Fes1 more negatively influenced Ssa1^{A17V}, reducing its intrinsic and stimulated ATPase activities to less than 30%. Fes1 had a minor but consistently reproducible positive effect on Ssa1^{R34K} intrinsic and Ydj1-stimulated ATPase, but slightly inhibited co-stimulation of Ssa1^{R34K} ATPase by combined Ydj1 and rCMLA. Fes1 positively regulated Ssa1^{L483W} ATPase, causing increases of 20% to 50% for the various assay conditions. Thus, Fes1 can act as both a positive and negative modulator of the Ssa1 ATPase cycle, and the direction and magnitude of effect on activity is influenced by the particular Ssa1 mutation.

Ssa1 mutants have altered protease digestion patterns

To determine if the Ssa1 mutations cause alterations in physical structure, we digested the proteins with proteinase K in the presence of ATP or ADP (Figure 5). Partial proteinase K digestions of various Hsp70s produce a 45 kDa fragment representing the NBD. In the ATP-bound state a portion of the adjacent SBD is protected producing a transient fragment of approximately 60 kDa [32,48,49]. For wild type Ssa1 typical digestion patterns were observed. In reactions with ADP the digest reached its final state in about 5 minutes and the main product was the characteristic 45 kDa NBD fragment (Figure 5A, band 3). Partial digestion in the presence of excess ATP showed the expected 60 kDa fragment (band 2).

In the presence of ADP all the Ssa1 mutants were similar to wild type, showing only the 45 kDa band after 5 minutes. In the presence of ATP, however, all three showed altered digestion patterns. For Ssa1^{A17V} and Ssa1^{R34K} the major 60 kDa product of the reaction seen for wild

type Ssa1 (band 2) was much fainter while a larger fragment (band 1) predominated. This shift reflects a change in structure that protects part of the SBD. The digestion patterns are similar, indicating both mutations have a similar effect, but protection in Ssa1^{R34K} persisted longer.

Ssa1^{L483W} was completely digested to the 45 kDa fragment (band 3) within 5 minutes even when ATP was present. We supposed this lack of protection was explained by the higher ATPase activity of Ssa1^{L483W} converting the protein to the quickly digested ADP bound conformation. However, very short duration digests of Ssa1^{L483W} (not shown) also failed to produce a 60 kDa fragment, raising the possibility that Ssa1^{L483W} does not adopt the normal ATP-bound conformation.

To test this possibility we repeated the protease digestion on ice (Figure 5). The ATPase activity of Ssa1^{L483W} is reduced by 12-fold at this temperature, making the hydrolysis rate similar to wild type Ssa1 at 25°. After only two minutes on ice there were two digestion products of Ssa1^{L483W}, the 45 kDa fragment and a faint protected fragment corresponding to the larger fragment (band 1) seen for the NBD mutants (Figure 5). Therefore, the rapid digestion of Ssa1^{L483W} is not explained by its elevated ATP hydrolysis converting it to the ADP-bound form. The L483W substitution must cause a structural alteration that prevents the protein from adopting the normal ATP-bound conformation. Thus, all three mutations cause conformational alterations that affect structure of the SBD or the interface between NBD and SBD.

Luciferase reactivation is enhanced for NBD mutants and reduced for the SBD mutant

To test the Ssa1 mutants in a functional assay, we monitored their ability to promote reactivation of denatured luciferase. Unfolded luciferase occupies a complicated series of transition states, only some of which can be reactivated [50]. The ability of chaperones to reactivate luciferase depends on the method of denaturation and the properties of the aggregates. Unfolded monomers and small aggregates can be rescued by combined Hsp70/Hsp40 (Ssa1/Ydj1), while larger more extensive aggregates require cooperation of a member of the Hsp100 family [28,51-54]. A precise abundance of the yeast Hsp100 homolog Hsp104 is essential for the propagation of [*PSI*⁺] [10], and a functional link between Ssa1 and Hsp104 regarding [*PSI*⁺] phenotype was established by genetics [55].

For reactions with only Hsp70 and Hsp40 (Figure 6A), luciferase was unfolded by mild heat treatment in the presence of Ydj1 and then diluted into a refolding reaction containing Ssa1. Luciferase was effectively inactivated (< 2% initial activity) in a form that could be considerably reactivated by Ssa1. All of the Ssa1 proteins reactivated luciferase to levels significantly above spontaneous refolding. However, compared with wild type, Ssa1^{A17V} and Ssa1^{R34K} refolded twice as much luciferase as wild type Ssa1, and Ssa1^{L483W} reactivated only about one third as much. Thus, prion-inhibiting Ssa1 mutations can either increase or decrease Hsp70 chaperone activity.

Restoration of extensively aggregated luciferase by combined Hsp104, Hsp70 and Hsp40 is shown in Figure 6B. All three chaperones were required to restore activity as no significant reactivation occurred when only Hsp104 or only the combination of Ssa1 and Ydj1 was present. Note that for reactions with Hsp104, luciferase was denatured differently and the final concentrations of reaction components were different, so the absolute numbers in Fig. 6A and Fig. 6B cannot be compared directly. Overall, however, the Ssa1 proteins cooperated with Hsp104 and Ydj1 to a degree that correlated with their function in the assay without Hsp104. The increased refolding by the NBD mutants is particularly pronounced at early time points. Wild-type and Ssa1^{L483W} reactivated luciferase linearly over the course of the assay but after 60 minutes total luciferase activity was still lower compared to reactions with Ssa1^{A17V} or Ssa1^{R34K}. As for ATPase stimulation and substrate binding, the NBD mutations increased luciferase disaggregation and refolding activities while the SBD mutation decreased them.

Thus, the way the mutations enhanced or inhibited function of the disaggregation machinery mirrored their effects on Ssa1 function. These data imply that function of Hsp104 disaggregation machinery depends on Hsp70 and suggest that anti-prion effects of the Ssa1 mutations might occur through alteration of the Hsp104 machinery.

Discussion

Our previous genetic screens identified many single amino acid mutations in the Hsp70 protein Ssa1 that similarly impair the strength and stability of the yeast prion [*PSI*⁺] [19]. Here we analyzed the enzymatic properties of Ssa1 mutants altered in both the same and different domains to determine if they share a common functional defect. We find each of the mutations altered Hsp70 activities distinctly. When taken together, however, our data suggest all of the mutants perturb communication between Hsp70 domains and all display some level of disconnect from wild type regulation of ATP hydrolysis and substrate interactions.

The NBD mutations A17V and R34K affected most Ssa1 functions similarly. Both caused increased peptide binding, substrate-stimulated ATPase, and ability to refold denatured luciferase. Although they are in the NBD, their effects are primarily in substrate interactions, demonstrating their influence on the strong inter-domain character of Hsp70 function. Consistent with this interpretation, their partial protease digestion patterns imply they have an altered conformation of the SBD or the NBD/SBD interface. A recent study of the endoplasmic reticulum Hsp70 BiP also showed that a mutation in the same lobe of the NBD alters interdomain communication and the protease digestion pattern of the SBD [56].

Although A17V and R34K similarly affected Hsp70 structure and function in general, subtle structural differences more dramatically affected specific functions. For example, while Ssa1^{A17V} was defective in synergistic stimulation of ATPase by Hsp40 and substrate, Ssa1^{R34K} behaved like wild type. Unexpectedly, this defect did not correlate with reduced luciferase refolding, which demonstrates the alteration distinctly affects interactions with different Hsp40/substrate pairs. The mechanism by which Hsp40 coordinates Hsp70 ATP hydrolysis with substrate capture is incompletely understood. Hsp40 interacts with both NBD and SBD of Hsp70 [57,58] and it has been suggested that substrate and Hsp40 compete for contacts with Hsp70 in a tri-protein complex [59,60]. Such interactions could influence specificity of Hsp70/Hsp40 pairs for particular substrates. In addition to affecting substrate interactions, A17V and R34K are in a lobe that interacts with Hsp40 and each might affect this specificity by altering interactions in this region.

The NBD and SBD of Hsp70s interact intimately and conformational changes in one domain are coordinated with activity in the other [61]. The substantially elevated intrinsic ATPase of Ssa1^{L483W} suggests the structure of its SBD is altered in a way that stimulates ATP hydrolysis in the NBD. In support of this interpretation we failed to observe the 60 kDa fragment normally seen after partial proteolysis of wild type Ssa1 even when ATP hydrolysis of Ssa1^{L483W} was reduced to the wild type rate. The mechanism of how the L483W substitution activates ATPase is unclear but the structure of the SBD is probably skewed to accommodate the bulkier side chain. Residue 483 is near two important regions involved in inter-domain regulation, the inter-domain α -A helix and the linker connecting the NBD and SBD. Structural changes caused by L483W could influence inter-domain contacts of this helix or dynamics of the linker, which undergoes dramatic movement during transition between ATP and ADP bound states and is important for activation of Hsp70 by Hsp40 [23,62].

The effect of Fes1 on the ATPase of Ssa1 was variable and showed that Fes1 can act as a negative and positive regulator. Fes1 is a homolog of the mammalian HspBP1 protein [63] and this class of NEF is structurally and mechanistically distinct from the GrpE and BAG-1 classes

of exchange factors [64]. HspBP1 can also negatively regulate Hsp70 [65,66]. Unlike DnaK of *E.coli*, Ssa1 can function normally in an *in vitro* refolding assay without an exchange factor [67,68] and the magnitude of the effects on ATPase by Fes1 is low compared to other Hsp70/NEF combinations such as DnaK/GrpE [45]. Thus, Fes1 may act more to fine-tune the activity of Ssa1 rather than as an essential part of the chaperone machinery.

Sse1 is another exchange factor for Ssa1 that has been shown to affect $[PSI^+]$ propagation [69-71]. Although none of the Ssa1 mutations are in a location predicted to affect interaction with Sse1, it has been shown that Sse1 binds to Ssa1 and can regulate its ATPase [72]. It is possible that the mutations affect the structure of the NBD in a way that alters this regulation.

While all the mutants appear to be altered in the coordination between NBD and SBD, we did not see a common change of any one specific activity. In fact, each of the three mutations distinctly affected Hsp70 function, and A17V and L4843W had very different and often opposite effects on Ssa1 activities. This disparity suggests there are many different ways Hsp70 function can be altered to disturb chaperone machinery function in a way that causes an amyloid-inhibiting effect. In line with the idea that an altered balance of Ssa1 activity is responsible for the antagonistic effects on the $[PSI^+]$ prion, the mutant Ssa1 proteins with the largest deviations from wild type activity display the weakest $[PSI^+]$ phenotypes. It was still very surprising to find the intrinsic ATPase activity of Ssa1^{L483W} to be so high because it supports cell growth as well as wild type Ssa1. This inconsistency implies that Hsp70 function or regulation in the cell is very different than in the test tube. Nevertheless, the fact that all of these mutant Ssa1 proteins support growth so well demonstrates that essential functions of Ssa1 are clearly more tolerant than $[PSI^+]$ to deviations in Hsp70 activity, and points to modulation of Hsp70 function as a method to influence amyloid growth *in vivo*.

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Figure 1.

Locations of $[PSI^+]$ -weakening Hsp70 mutations. (A) Locations of Ssa1 mutations are indicated on the ribbon structure of the *E. coli* Hsp70, DnaK created using Rastop software. The left panel shows the isolated nucleotide-binding domain (NBD). Arrow indicates the ATP binding cleft. The A17V residue is shown in blue and R34 in cyan on the right lobe of the NDB. The right panel shows the substrate-binding domain (SBD) with residue L483 in red. The black ribbon represents peptide substrate in the binding pocket. The lid region over the substrate-binding pocket and the important inter-domain α -A helix and linker region are indicated.

Figure 2.

Ssa1 proteins and their ATPase and peptide-binding activities. (A) Purified samples of Ssa1 proteins used for ATPase measurements. Proteins (2.5 and 5.0 μ g of each) were stained by Coomassie blue after SDS-PAGE. (B) Intrinsic and substrate-stimulated steady state ATPase activities of Ssa1 proteins. ATP turnover (mole/min/mole) of purified wild type (WT) and mutant (as indicated) Ssa1 proteins are shown as mean $\pm$ SD of five measurements. Activities are intrinsic (Int) or for proteins incubated with rCMLA or A7 peptide substrate (40 or 80 μ M as indicated). Numbers over the bars indicate the fold stimulation over intrinsic levels. Note that the scale of the upper part of the graph is compressed. Intrinsic activities of Ssa1^{A17V} and Ssa1^{R34K} are not statistically different from WT ($P < 0.2$) while Ssa1^{L483W} is significantly

different from WT ($P < 0.0001$). The stimulated activity of all three mutants is significantly different from the stimulated activity of WT ($P < 0.02$ for rCMLA and $P < 0.01$ for A7 peptide). (C) Substrate binding of Ssa1 proteins. Ssa1 proteins (1 or 2 μM as indicated) were added to fluorescein labeled A7 peptide and the fluorescence anisotropy was measured after 1 hr. Anisotropy of the peptide without added Ssa1p was subtracted from each reaction. Values are mean $\pm$ SD of three measurements. Binding of all three mutants is significantly different from WT ($P < 0.01$ at 2 μM protein).

Figure 3.

Stimulation of Ssa1 ATPase by Hsp40. (A) Steady state ATPase for wild type and mutant Ssa1 proteins incubated with a 4-fold molar excess of Ydj1 or Sis1. Values are mean $\pm$ SD of at least three measurements. Numbers over the bars indicate the fold stimulation by each Hsp40. Hsp40 stimulated activity of Ssa1^{A17V} and Ssa1^{R34K} is not significantly different from Hsp40 stimulated activity of WT ($P < 0.3$) while Ssa1^{L483W} stimulated activity is significantly different from WT ($P < 0.01$) (B) ATPase activities of Ssa1 proteins without Ydj1 (0) and with Ydj1 at molar ratios from 0.25 to 4-fold. Values are averages of two measurements at each concentration. (C) Steady state ATPase of Ssa1 proteins stimulated by Hsp40 with and without peptide substrate. Values are mean $\pm$ SD of three measurements with 4-fold molar excess Ydj1

(Y) or Sis1 (S) alone, or with addition of 40 μ M rCMLA substrate (Y/c and S/c). The numbers over the bars indicate the fold stimulation by addition of rCMLA. WT and Ssa1^{R34K} were significantly stimulated over Ydj1 alone by addition of rCMLA ($P < 0.0001$) while Ssa1^{A17V} and Ssa1^{L483W} were not ($P < 0.1$). Addition of rCMLA did not further stimulate WT, Ssa1^{A17V}, or Ssa1^{R34K} over Sis1 alone ($P < 0.3$) but did stimulate ^{L483W} ($P < 0.03$).

Figure 4.

Effect of nucleotide exchange factor Fes1 on ATPase of Ssa1 proteins. Steady state ATPase activities of Ssa1 proteins in reactions with Fes1 are shown relative to their activities in identical reactions without Fes1, which are normalized at 100. Reactions contained no other additions (Int), Ydj1 (Y), or both Ydj1 and CMLA (Y/c). Concentrations were 0.4 μ M Ssa1, 0.8 μ M Ydj1, 40 μ M rCMLA, and 0.8 μ M Fes1. Values are mean $\pm$ SD of at least three measurements.

Figure 5.

Protease digestion of Ssa1 proteins. (A) Ssa1 proteins were incubated on ice with 5 mM ATP or ADP and then digested with proteinase K for 0, 5, or 15 minutes at 25°. Proteins were separated by SDS-PAGE and stained with Coomassie blue dye. Arrows indicate positions of the 3 major reaction products. To determine if the ADP-like digestion pattern of L483W in the presence of ATP was due to its high intrinsic ATPase, additional protease reactions containing Ssa1^{L483W} and 5 mM ATP were incubated on ice to reduce ATPase activity to a level similar to the other proteins. The graph shows steady state ATPase of Ssa1^{L483W} at 25° or 4°.

Figure 6.

Refolding of denatured luciferase. (A) Refolding by Ssa1 and Ydj1. Luciferase enzyme was denatured at 42° for 15 minutes, then diluted into a refolding reaction containing Ssa1 and Ydj1. At the indicated times aliquots were removed and luciferase activity was measured. (B) Refolding by Hsp104, Ssa1, and Ydj1. To cause more extensive aggregation luciferase was unfolded in urea and diluted into cold buffer before adding to chaperones. Values are mean $\pm$ SD from at least five separate refolding reactions. Luciferase refolding of all three mutants was significantly different from WT at the conclusion of the assay ($P < 0.02$).