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Mutations in the yeast Hsp70, Ssa1, at P417 alter ATP cycling, interdomain coupling, and specific chaperone functions

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Abstract

The major cytoplasmic Hsp70 chaperones in the yeast *Saccharomyces cerevisiae* are the Ssa proteins, and much of our understanding of Hsp70 biology has emerged from studying *ssa* mutant strains. For example, Ssa1 catalyzes multiple cellular functions, including protein transport and degradation, and to this end the *ssa1-45* mutant has proved invaluable. However, the biochemical defect(s) associated with the corresponding Ssa1-45 protein (P417L) are unknown. Consequently, we characterized Ssa1 P417L as well as a P417S variant, which corresponds to a mutation in the gene encoding the yeast mitochondrial Hsp70. We discovered that the P417L and P417S proteins exhibit accelerated ATPase activity that was similar to the Hsp40-stimulated rate of ATP hydrolysis of wild type Ssa1. We also found that the mutant proteins were compromised for peptide binding. These data are consistent with defects in peptide-stimulated ATPase activity and with results from limited proteolysis experiments, which indicated that the mutants' substrate binding domains were highly vulnerable to digestion. Defects in the reactivation of heat-denatured luciferase were also evident. Correspondingly, yeast expressing P417L or P417S as the only copy of Ssa were temperature sensitive and exhibited defects in Ssa1-dependent protein translocation and misfolded protein degradation. Together, our studies suggest that the structure of the substrate binding domain is altered and that coupling between this domain and the nucleotide binding domain is disabled when the conserved P417 residue is mutated. Our data also provide new insights into the nature of the many cellular defects associated with the *ssa1-45* allele.

Graphical abstract

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Keywords

molecular chaperone; protein folding; protein degradation; protein translocation; ERAD; temperature-sensitive

Introduction

Protein function depends upon the ability of a polypeptide chain to attain its three dimensional conformation. While small proteins may spontaneously achieve their native structures, the folding of larger, more complex proteins and particularly membrane proteins is an error-prone process. Thus, all cells harbor factors known as molecular chaperones that facilitate protein folding either by preventing the acquisition of off-pathway intermediates (e.g., aggregates) or by directly augmenting specific steps in the folding pathway [1–4].

One universally conserved chaperone family is the Hsp70s (heat shock proteins of 70 kDa). In addition to preventing aggregation of newly synthesized or unfolded proteins, Hsp70 chaperones are integrally linked to critical cell activities, including protein translocation across membranes, the identification of misfolded proteins and the targeting of these substrates to degradation pathways, and the control of gene expression. Because of these essential functions, Hsp70 activity is also associated with diseases such as cancer and neurodegenerative disorders [5–8]. In some cases, the modulation of Hsp70 activity has proved effective in lessening disease presentation in model systems [9,10].

Hsp70s are constructed from a conserved two domain structure: the nucleotide binding domain (NBD) and the substrate binding domain (SBD) [11]. The NBD associates avidly with adenosine nucleotides and is an inherently slow ATPase. However, this activity can be modulated by Hsp40 co-chaperones [12]. Hsp40s, which are also known as J domain-containing proteins, help specify and regulate Hsp70 function [13]. In addition, Hsp70 activity is regulated by nucleotide exchange factors (NEF), which accelerate the release of ADP, favor ATP binding, and thus speed up the ATP hydrolytic cycle [14]. In contrast, the SBD interacts with peptides and even with some globular proteins to exert Hsp70-dependent activities. This is accomplished through a cleft in the SBD, which accommodates short amino acid peptides with hydrophobic character. The SBD also harbors a C-terminal series of α -helices that close over the substrate binding cleft and prevent substrate release [15].

The conformation of the SBD and the position of the α -helices are influenced by the nucleotide bound state of the NBD. When the NBD is bound to ATP, the SBD resides in a more open state, allowing rapid substrate exchange [16]. After ATP is hydrolyzed the SBD

adopts a more closed structure that prevents substrate dissociation. In turn, the two domains are connected by a flexible linker, which helps cement the domains in the ATP-bound state and is important for interdomain communication, which in turn is regulated by the ATP hydrolytic cycle [17,18].

The yeast *Saccharomyces cerevisiae* has served as a valuable model organism to unravel the functions of specific proteins in eukaryotic cells. Our current knowledge of the cellular activities of Hsp70s, Hsp40s and NEFs highlights this fact. For example, the Ssa family of Hsp70s consists of four homologous cytosolic proteins, Ssa1–4 [19], which possess overlapping function, although select activities have been ascribed to one but not another Ssa family member [20,21]. Nevertheless, the expression of any single Ssa protein is sufficient to support viability. Thus, by deleting the other three family members, the essential functions of a lone Ssa protein can be studied and the effects of specific mutations investigated. A similar analysis in human cells would prove arduous, given the existence of six distinct Hsp70 homologues in the cytosol [22].

An *SSA1* temperature sensitive allele, *ssa1–45*, was first described by Craig and colleagues in 1996 as exhibiting a defect in the translocation of a yeast mating pre-pheromone, prepro α -factor (ppaf), into the ER [23]. Genetic interactions between this allele and an Hsp40-encoding gene, *YDJ1*, were also uncovered. The *ssa1–45* allele has subsequently been used to define other diverse Ssa1 (and thus Hsp70)-dependent functions, including thermal tolerance, endoplasmic reticulum associated degradation (ERAD), transcriptional control, ribosome integrity, protein translation, protein aggregation, and protein folding (see for example [24–32]). Despite extensive use of the *ssa1–45* strain, the Ssa1–45 protein has never been characterized. While yeast cells carrying *ssa1–45* as the sole Ssa1 protein fail to propagate at 37°C and demonstrate numerous cellular defects at elevated temperatures, it is unknown if this phenomenon arises due to a temperature dependent loss of protein activity, and if so, which of the many Hsp70-requiring functions are compromised. Alternatively, the activity of the mutant protein may be altered compared to the wild type level chaperone at the non-permissive temperature, thus exerting chaperone-specific defects.

The *ssa1–45* allele bears a single amino acid change at position 417 from Pro to Leu. The presence of a Pro at or near this position in diverse Hsp70s, including the yeast Ssa2–4 proteins, the related but functionally distinct Ssb1–2 proteins, the Hsp70-like Sse1–2 NEFs, and the mitochondrial Ssc1 protein, as well as *E. coli* DnaK and the bovine and human heat shock cognate proteins of 70 kDa (Hsc70), suggest the important nature of this residue. Proline is well known to rigidify the polypeptide chain and significantly affects secondary structure [33]. In fact, a previously described temperature sensitive mutant allele of the yeast mitochondrial Hsp70, *ssc1–2*, contains a Pro to Ser mutation at position 442 [34]. Like *ssa1–45*, the *ssc1–2* allele has been used to study several aspects of mitochondrial biology. For example, yeast containing the *ssc1–2* allele are defective for both mitochondrial protein translocation and folding [35–37].

In this study, we report on the biochemical activities of the Ssa1–45 (P417L) protein along with a new Ssa1 mutant protein that contains the *ssc1–2*-like substitution of a Pro to a Ser at residue 417 (P417S). Our results demonstrate similar thermal stability of the P417 mutant

proteins and wild type Ssa1 in the presence of ATP. However we noted prominent defects in essential biochemical activities, even at permissive and semi-permissive temperatures. While both the P417L (Ssa1–45) and P417S proteins exhibited similar defects, the P417S change was generally less pronounced. However, yeast expressing P417L or P417S as the only copy of Ssa were equally compromised for cell growth and specific Hsp70-dependent functions at semi-permissive and non-permissive temperatures. Thus, we posit that essential activities are compromised below a critical threshold in the P417L- and P417S-expressing cells. Overall, our efforts will aid in interpreting the many papers in the literature that have employed the *ssa1–45* strain, which combined have helped decipher the myriad roles played by Hsp70 in the cell.

Results

The Ssa1–45 Pro-to-Leu substitution at position 417 resides in the substrate binding domain and may alter functions associated with the substrate binding domain and interdomain coupling

The Pro at position 417 was modeled onto the structure of *E. coli* DnaK (see Materials and Methods) and is shown in Fig 1. Residue 417 is located in the SBD and is in the loop between β strands 2 and 3. As noted in the Introduction, P417 is conserved in diverse Hsp70s, suggesting that it helps confer structural integrity. Based on the position of this residue, we predicted that mutations might alter the conformation of the SBD by disrupting the turn in the loop between the β strands that form the base of the hydrophobic peptide binding pocket. This could have direct effects on substrate binding. Based on studies of the DnaK protein, mutations in this loop disrupt interdomain communication between the SBD and NBD [38], leading to a loss of regulation of the ATP hydrolytic cycle. Additionally, an altered SBD conformation might impact the movement of the interdomain linker. Dynamic movement of the linker takes place during the transition between the open and closed conformations of the SBD [39], and the importance of this linker in maintaining Hsp70 function has been demonstrated [18].

P417 mutants exhibit higher rates of ATP hydrolysis and a greater dependence on nucleotide exchange factors

To assess how mutations at P417 affect Hsp70 function, we purified wild type Ssa1 and Ssa1 containing the P417L and P417S mutations from yeast to >98% purity (see Materials and Methods). No additional non-Ssa1 bands were visualized in the purified protein samples. Circular dichroism measurements of all three proteins indicated a mixed α - β protein. The spectra were similar, suggesting that the mutations do not cause gross alterations of protein structure (Supplemental Figure 1). However, both mutants displayed a slightly larger absorbance at 208 nm, suggesting that the structures of the proteins might be subtly altered and that the relative α -helical content might be greater. Next, we measured the steady state ATPase activities of the purified proteins in the presence or absence of a known peptide substrate or of J domain-containing co-chaperones that interact with Ssa1. As shown in Fig. 2a, the basal ATPase activity of the mutant proteins was considerably higher than wild type Ssa1. The average increased ATPase activity of the P417L and P417S isolates was 5–6 fold greater than wild type Ssa1 with P417L exhibiting a slightly higher activity than

P417S. It is not uncommon to observe accelerated ATP hydrolysis in Hsp70 mutants, which has been reported in numerous other studies (e.g., [17,38,40,41]).

The ATPase activity of Hsp70 chaperones is significantly stimulated by J domain-containing Hsp40 co-chaperones and is more modestly stimulated by substrate binding [42]. Previous work indicated that a select group of Hsp40s interact with Ssa1 and robustly enhance ATP hydrolysis, including Ydj1 and Hlj1 [43–46], and that a peptide, ala-p5, stimulates the ATPase activity of Ssa1 [47]. Therefore, we supplemented the assays with purified Ydj1, with a fusion protein containing the J domain of Hlj1, or with ala-p5 (Fig. 2b). When either the J domain of Hlj1 or full length Ydj1 were added at a 2:1 molar ratio to Ssa1, we observed the expected stimulation of Ssa1's ATPase activity by 4–6 fold over the unstimulated activity [48]. Addition of the ala-p5 peptide (at a large excess concentration of 125 μ M) resulted in an ~2.5-fold stimulation of wild type Ssa1, in-line with the level of stimulation seen when Hsp70s bind hydrophobic peptides [49]. In contrast, both Ssa1 mutants were only minimally stimulated (<2-fold) by a 2:1 molar ratio of J domain protein to Ssa1. Similarly, the ATPase of the P417L mutant was not stimulated by the peptide substrate, whereas a slight (~20%) increase in activity was evident when the ala-p5 peptide was added to the P417S variant.

To investigate further the nature of the defect(s) associated with the mutation of Pro at position 417, we performed single turnover assays with each of the three Ssa1 proteins to monitor the isolated hydrolysis step in the ATPase cycle. The protein-nucleotide complexes were spotted onto thin layer chromatography (TLC) plates immediately after isolation and the ADP resolved from ATP is visualized in Supplemental Figure 2. When the total radioactivity of the protein/nucleotide complexes was adjusted for protein concentration, we found that P417L and P417S incorporated only 60% and 83% of the nucleotide, respectively, relative to wild type Ssa1. This result demonstrated a difference in nucleotide interaction with the mutants that could arise from compromised binding and/or from faster nucleotide release (also see Fig. 4, below). Reflective of the higher ATPase activity of the mutants, ATP was partially hydrolyzed to a greater extent in the isolated complexes containing the mutant proteins: 52% ATP for P417L, 75% ATP for P417S, and 93% ATP for the wild type chaperone.

Next, aliquots of snap-frozen complexes were quickly thawed and incubated in the presence or absence of 1 μ M Ydj1, or 100 μ M ala-p5. Samples were removed every 20 sec and the products were again resolved by TLC. The loss of ATP in this experiment is presented in Fig. 3a–c. and the appearance of ADP over the time course is shown in Fig. 3d–f. The data were fit to a single exponential decay and the calculated rates of hydrolysis were $1.06 \times 10^{-3} \text{ s}^{-1}$ (wild type), $1.07 \times 10^{-2} \text{ s}^{-1}$ (P417L) and $5.35 \times 10^{-3} \text{ s}^{-1}$ (P417S). These values are comparable to the turnover rates we observed in the steady state assays. Also consistent with our steady state results, these single turnover studies demonstrated that both of the ssa1 mutants showed accelerated ATP hydrolysis relative to the wild type protein. We also confirmed that the Hsp40 Ydj1 and the ala-p5 peptide significantly increased the hydrolysis of ATP of wild type Ssa1, whereas very little effect was observed on the hydrolysis rate of the mutants in the presence of these added peptides/proteins.

One explanation for the higher ATPase activities of the P417 mutant forms of Ssa1 is that they adopt a conformation that mimics the co-chaperone-stimulated state. Alternatively, deletion of the SBD may result in an isolated NBD and linker that exhibits accelerated ATPase activity; select mutations in the linker and in the SBD also lead to increased ATP hydrolysis [18,38,50]. Thus, alterations at P417 might give rise to a protein in which the NBD-SBD interface (and possibly the linker) is disrupted (see above and Fig. 1). Such a disruption of interdomain contacts might release the “braking” effect that the SBD can exert on the ATPase activity of the NBD in the absence of a bound substrate.

We next tested the effect of a known Ssa1-interacting NEF, Fes1, on the steady state ATPase activity of wild type Ssa1 and the P417 mutant proteins. Previously, Fes1 was reported to either inhibit or stimulate the steady state ATPase of Ssa1 and the effect was highly sensitive to the concentrations employed and to the assay conditions [51,52]. Under the conditions of our assay, wild type Ssa1 ATPase activity was mildly stimulated by GST-Fes1 (20%; Fig. 4a). In contrast, Fes1 more strongly accelerated the ATPase activity of P417L and P417S. In particular, the P417L protein was greatly stimulated by Fes1 in a dose dependent manner. These data most likely reflect the increased time that the chaperones spend in the ADP bound conformation and are consistent with the inherently accelerated rate of ATP hydrolysis; i.e., in contrast to the wild type protein, the rate-determining step for the P417L and P417S proteins is nucleotide release, at least under the conditions used in these assays.

We next measured the effect of Fes1 on the amount of bound ADP for the wild type protein versus the P417L and P417S mutants. First, radiolabeled ATP complexes with the chaperones were formed and separated from unbound nucleotide by gel filtration. To ensure that nucleotide release was not influenced by different ratios of ATP/ADP, the complexes were allowed to incubate until they contained almost exclusively ADP, as assessed by TLC (see Materials and Methods and data not shown). The complexes were then incubated with Fes1 or buffer. Ssa1 was then rapidly purified away from released nucleotide by centrifugation through G50 spin columns, and the remaining bound nucleotide was quantified by scintillation counting. As shown in Fig. 4b., Fes1 enhanced the release the ADP from all three proteins. As seen in Supplemental Figure 2, the P417L nucleotide complex had significantly less bound nucleotide than wild type Ssa1, and lost more nucleotide even in control reactions (Fig. 4b.), which lowered the apparent effect of Fes1. Together, these results suggest that P417L may bind nucleotide less avidly and/or has a naturally faster release rate even in the absence of a NEF.

The P417 mutant proteins and wild type Ssa1 exhibit similar thermal stabilities in the presence of nucleotides

Although the data presented thus far indicate substantial defects in P417L and P417S function at permissive temperatures, the proteins may become less stable at elevated temperatures, thereby giving rise to the *in vivo* temperature sensitive defect. Therefore, to test the thermal stability of wild type Ssa1 and the P417L and P417S proteins we quantified protein binding to a hydrophobic fluorescent dye, SYPRO Orange, during thermal denaturation [53]. In the presence of either ATP or ADP, no significant difference in thermal

stability was seen between the wild type and mutant proteins. The midpoint transition temperature with ATP or ADP present at a final concentration of 500 μ M is summarized in Table 1 and a representative curve is shown in Supplemental Figure 3. The thermofluor binding assay did not reveal multiple transition peaks, as has been seen using differential scanning calorimetry [38,54,55]. Instead, only one transition is evident, as previously demonstrated using this method [56]. The slope of the transitions was qualitatively similar for all three proteins and no significant shift in the curve was seen for the mutant proteins compared to wild type Ssa1. Combined with the results presented above, a mutation of the Pro at position 417 alters the biochemical activities of Ssa1 even at permissive temperatures and loss of cell viability at non-permissive temperatures may not be explained by thermal instability. Instead, a critical loss of an essential chaperone function most likely becomes evident at the non-permissive temperature.

The Ssa1 P417L and P417S mutant proteins are peptide binding-deficient and defective for refolding a denatured protein substrate

As shown in Fig. 2b and Fig. 3, an Ssa1 peptide substrate stimulated the ATPase of wild type Ssa1 but had no or little effect on P417L and P417S activity. To investigate if the P417 mutants are peptide-binding defective we titrated a fluorescently labeled version of the ala-p5 peptide into reactions containing the mutant proteins as well as wild type Ssa1. Binding was measured by the change in fluorescence anisotropy, as described previously [57], and measurements were initially taken after 4, 6, and 24 hour incubations. These pilot experiments indicated that binding equilibrium was established after 24 hours, and in another series of controls we observed that peptide binding was competed with excess unlabeled peptide and was released upon the addition of ATP (data not shown). Next, the results from two independent titrations were averaged, data were taken from the 24 hour time point, and the resulting binding curves are shown in Fig. 5a. A K_D calculated for the wild type protein was 71 ± 2.8 nM, which is in line with the known binding affinity of Hsp70s for small hydrophobic peptides [41,58]. Based on these data, it is clear that both mutants bound considerably less peptide than wild type Ssa1. Consistent with these data, we also discovered that the mutant proteins were unable to bind a radiolabeled form of reduced carboxymethyl lactalbumin, as assessed using native gel electrophoresis [59](data not shown). Thus, the mutations at P417 alter substrate interaction.

It was formally possible that the mutant proteins aggregated or were partially degraded during incubation, which would result in an apparent loss of peptide-binding and/or a lower peptide affinity. To examine this scenario, we incubated each protein under the same conditions used in the binding reaction (4° C for 24 hours). At the end of the incubation we subjected the mixture to centrifugation and the supernatant was removed. We then analyzed equal amounts of sample before incubation and we analyzed the load and supernatant and pellet fractions after overnight incubation. The data shown in Fig. 5b demonstrate that there was no degradation apparent over the course of the incubation and that the protein remained in the supernatant, indicating the absence of protein aggregation.

Because the inherent ATPase activity of the mutants was accelerated and refractory to the addition of an Hsp40 co-chaperone, and because peptide binding efficiency and affinity

were significantly compromised, we predicted that the P417L and P417S mutants would also be unable to refold denatured firefly luciferase (FFlux). The refolding of FFLux is an accepted assay to monitor chaperone function. To test this hypothesis, we subjected FFLux to a mild heat shock, which inactivated the enzyme but retained the protein in a form that could be reactivated when added to Ssa1 and the Ydj1 co-chaperone. The restoration of FFLux activity after incubation with Ssa1 and Ydj1 is shown in Fig. 6a,b. Wild type Ssa1 and Ydj1 at a final concentration of 1 μ M resulted in significant enzyme reactivation (~26 % of the starting activity at 60 minutes), whereas refolding was impaired when the Ssa1 mutants were added in the presence of Ydj1 at these concentrations. P417L was particularly defective as only 3% of FFLux was reactivated compared to wild type Ssa1 at the 60 min time point. Supplementation with additional chaperone improved refolding and P417L reactivated 14% of wild type activity at 2 μ M. Of interest, the P417S mutant was ~4-fold better than P417L at reactivating FFLux (at 2 μ M each), but at 2 μ M this mutant was still only able to reactivate 58% of FFLux compared to the wild type control when present at 1 μ M. We further note that there was no significant reactivation over background by Ydj1 alone.

The substrate binding domain of the P417 mutant proteins is more protease-sensitive than wild type Ssa1

The data shown in the preceding sections indicate that the P417S and P417L mutants display various chaperone-dependent defects and that distinct structural perturbations may be evident. To further explore this hypothesis, we treated each protein with limiting amounts of proteinase K for 0–10 min at 30°C and in the presence of excess ADP. The digested fragments were then analyzed by SDS-PAGE and staining with Coomassie blue. As shown in Fig. 7, wild type Ssa1 was rapidly clipped to a slightly smaller form, which represents a species lacking the C-terminal lid. Over time, this was followed by the appearance of a ~45 kDa form that represents the free NDB after complete digestion of the SBD. Indeed, numerous studies indicate that the 45 kDa NBD of Hsp70 is protease resistant whereas the SBD is proteolytically labile (see for example [60–62]). In contrast, both the P417S and P417L mutants were quickly digested to the 45 kDa species. Thus, the mutation of a Pro at position 417 alters Ssa1 structure in a way that exposes protease sites in the SBD, which is indicative of a conformational change in this domain. However, wild type Ssa1 was more stable than either mutant, demonstrating that even in the ADP bound, closed conformation the structure of the mutants is perturbed, resulting in greater accessibility of a protease-sensitive site.

Yeast expressing Ssa1 P417 mutants are temperature sensitive and exhibit Hsp70-associated defects

To determine the effect of the P417 mutants on yeast cell growth and Ssa1-dependent activities, we constructed strains expressing wild type Ssa1 or the P417L or P417S Ssa1 mutant proteins as the only Ssa family member (see Materials and Methods). Each strain was then serially diluted onto medium and the plates were incubated at 26°C, 30°C, 34°C, or 37°C for 3 days. As evident from the data presented in Fig 8a, the phenotype of the two mutant strains was essentially identical: Wild type Ssa1 supported growth at 37°C, whereas neither mutant strain was able to do so. Both mutants also grew slower at 34°C and showed

significantly reduced growth relative to wild type, a phenotype that was less pronounced at lower temperatures. We also observed that the mutants recovered from stationary phase more slowly than the wild type strain (data not shown). These assays demonstrate that mutating Pro 417 to Ser or Leu has similar effects on cell growth, even though select biochemical differences were noted between the P417L and P417S proteins. Consistent with a similar effect on cellular homeostasis, the steady state expression of the P417L and P417S proteins was considerably higher than that of wild type Ssa1 (Fig. 8b); therefore, yeast expressing the mutant proteins induce a heat shock or stress response. (In this experiment, yeast were grown at 26°C and then the cells were shifted to the temperatures used for the spot assays for 2 hr.) Notably, even at permissive temperatures there is a 3–4 fold induction of the mutant proteins. This result is in-line with the data, presented above, that the corresponding proteins exhibit biochemical defects at permissive temperatures. We note that the difference between the levels of the wild type and mutant strains was less pronounced at higher temperatures, which arises due to increased (~2-fold) wild type expression at 34°C and 37°C.

Ssa1 is required for the ERAD of misfolded membrane proteins and *ssa1-45* (P417L) yeast have been used to define Ssa1-dependent defects in ERAD at non-permissive temperatures [63–68]. To investigate whether the P417S mutant was also ERAD defective we performed cycloheximide chase assays at 37°C using a model Ssa1-dependent ERAD substrate, Ste6*. Ste6* is a truncated version of the yeast α -factor mating pheromone transporter, Ste6, and is retained in the ER and fails to transit to the plasma membrane where it normally functions [69]. While Ste6* is degraded rapidly and completely in strains expressing wild type Ssa1, degradation is severely compromised in the *ssa1* mutants (Fig. 9a). Even at 34°C, Ste6* degradation was still blunted in the mutant strains relative to wild type cells, and again no major differences were seen between the degradation profiles corresponding to the P417L and P417S-expressing cells (data not shown).

We next assessed the ability of the *ssa1* mutant yeast strains to support the translocation of ppaf into the ER. The *ssa1-45* (P417L) allele confers a translocation defect [23], and we recapitulated this phenomenon in cells grown at 26°C and then shifted to 37°C (Fig. 9b). The same result was observed in the P417S-expressing strains. Therefore, only the P417L and P417S mutant yeast strains accumulated the untranslocated ppaf species at the nonpermissive temperature. The greater accumulation of the paf species in the mutant strains is likely due to the ERAD defects noted above. Together, these assays demonstrate that yeast expressing either P417L or P417S compromise Hsp70-catalyzed events at the ER membrane.

Discussion

In this paper, we delineate the biochemical defects of a protein linked to a long-employed genetic “tool”, the *ssa1-45* (Hsp70) mutant yeast strain. For a mutant that was discovered in a screen for a temperature dependent loss of function, it might be envisioned that the purified Ssa1 mutant protein, Ssa1-45 (P417L), would show decreased thermal stability *in vitro*. Surprisingly, when bound to either ATP or ADP the thermal stability was the same as the wild type protein. Our deeper biochemical characterization of the P417L and P417S

proteins instead demonstrated altered biochemical activities, even at permissive temperatures.

The structure of the Hsp70 SBD is a two layered β -sandwich, which undergoes conformational rearrangements to transition between the open and closed conformations [70]. Recent structural studies show that not just the lid, but the entire β structure of the SBD undergoes dramatic movement. The β strands rearrange and the substrate binding pocket expands and contracts. Residue 417 is located in the loop between β -strands 2 and 3. This loop connects the upper and lower β sheets of the substrate binding pocket, and β strands 1 and 2 as well as loops L1,2 and L3,4 contact the backbone of the bound substrate [70]. Upon ATP binding there is movement of loops L2,3, L4,5, and L6,7 into positions that engage the NBD [16]. These rearrangements of the β strands dictate how ATP allosterically couples the SBD and NBD, thereby regulating substrate binding and triggering ATP hydrolysis [71]. Not surprisingly, mutations in residues within this region disrupt SBD-NBD coupling [72–74]. Therefore, mutations at P417 are in a location that could be expected to influence both the substrate binding pocket as well as communication between the SBD the NBD. Evidence presented here suggests that both mechanisms may contribute to the altered function of the Ssa1 mutants and may explain why the P417S mutant was less affected than the P417L mutant in select assays.

The most obvious effect of the P417 mutations is a significant acceleration of the steady state ATPase activity over the otherwise slow basal rate (0.07 min^{-1}) and greatly decreased stimulation by Hsp40; similar results were evident in single-turnover measurements. A hallmark of normal Hsp70 activity is the tight coupling of ATP hydrolysis to substrate capture, most often in cooperation with a J domain-containing co-chaperone, such as an Hsp40 [75]. The higher basal activity of the P417L and P417S proteins and the reduced stimulation seen upon Hsp40 addition suggests that the mutant Hsp70s may interact with an Hsp40, but ATPase activity is not further stimulated since the chaperones are already activated. Similar effects were seen when other Hsp70 point mutants have been examined [18,34,41]. It is known that the SBD acts as a brake on NBD's ATPase activity and that the free NBD with the linker domain attached has a higher activity than full length Hsp70 [76]. It is thought that in the absence of substrate the SBD stabilizes the NBD and prevents ATP hydrolysis [18,50]. As suggested above, these data are in-line with the notion that P417 plays an important role in mediating interdomain contacts between the NBD and the SBD, which regulates ATP hydrolysis. Therefore, it is possible that mutations at P417 disrupt an interaction between the two domains that removes the braking effect of the SBD [77]. There is also evidence that Hsp40 proteins interact not only with the NBD but also contact the SBD [78–80]. A change in structure that mimics the interaction of J domain-containing co-chaperones may, in theory, place the Ssa1 mutants in a permanently stimulated state.

Based on our data indicating that the SBDs of the mutant proteins are hypersensitive to exogenous protease, and based on the fact that mutating P417 led to peptide-binding defects, it is likely that loss of the Pro in the loop between β strands 2 and 3 skews the SBD. Remarkably, a previous screen using *E. coli* DnaK to isolate mutants with reduced peptide binding uncovered the P419L and P419S mutations, which are equivalent to our mutations in the Ssa1 gene [81]. The authors postulated that the sharp turn of loop L2,3 requires Pro to

be in the *cis* conformation. Other residues in this loop have also been associated with interdomain coupling. For example, both K414 and N415 in the SBD make contacts with the NBD when DnaK is in the open conformation [82]. Another revealing study [38] showed that the DnaK K414I mutation exhibits similar effects as the P417L mutation in Ssa1. In this prior study, K414I had a higher ATPase activity, which was generally refractory to the addition of DnaJ or a peptide substrate. A reduced affinity for peptide (from 60 nM to 400 nM) was also measured, which is qualitatively similar to what we see for the P417 Ssa1 mutants. In addition, the DnaK K414I mutant lacked intrinsic tryptophan quenching that is normally evident in wild type DnaK upon ATP binding. This result was interpreted as the acquisition of an abnormal SBD-NBD interface. Finally, it was reported that K414 in DnaK is a sensitive protease site [60], consistent with our observation that changes in this loop lead to increased protease digestion. In light of the recent information showing an intimate association of NBD and SBD in the ATP bound open conformation (see above), it is clear that an alteration of the secondary structure in and around the L2,3 loop where residue 417 is located could have strong effects on the interface between the two domains. Overall, we believe that mutating P417 in the SBD of Ssa1 alters contacts with the NBD and results in a loss of allostery between the two domains. Consequently, ATPase activity is accelerated, Hsp40 regulation is lost, and the deregulation of lid closing and opening impacts efficient substrate capture. Another non-mutually exclusive possibility is that the position of the hydrophobic linker has been distorted and the loss of normal contacts between the linker and surrounding residues leads to a similar loss of interdomain communication. Based on the structure presented in Fig. 1, we favor the first scenario, but either of these models would explain why FFlux refolding was inhibited in the presence of the P417L and P417S mutants. Definitive proof of these conjectures will require a more involved and refined structural analysis of the Ssa1 mutants.

The ATPase activity of the P417S mutant was generally similar to the P417L mutant, indicating that mutating the Pro at position 417 uncouples allosteric communication between the SBD and NBD. Digestion with low concentrations of proteinase K in the presence of excess ADP revealed that the NBD of both mutants was produced more rapidly, indicative of a conformational change in the SBD that leads to greater exposure of a protease-sensitive site.

While the P417L and P417S mutations similarly affect Ssa1 they are not identical. Compared to the P417S mutant, the P417L chaperone had less bound nucleotide after the purification of protein/nucleotide complexes, lost more nucleotide in our nucleotide release assays, and was stimulated to a greater extent by the Fes1 NEF in the steady state ATPase assay. The P417L also displayed a larger increase in ATP hydrolysis than P417S in single turnover assays, suggesting that both the hydrolysis and release step of the ATPase cycle might be altered more dramatically.

While the P417S mutant was less efficient than wild type Ssa1 at reactivating FFlux, it was clearly more effective than P417L. This leads us to speculate that the smaller, less hydrophobic side chain (Ser versus Leu) does not distort the local secondary structure to the same extent and therefore may not alter the conformation of the substrate binding pocket as dramatically. However, the two mutants were similarly defective at binding a short, model

peptide *in vitro*. This apparent discrepancy is not so surprising given that recent structural studies have demonstrated that a unique conformation is evident when the SBD binds small peptides compared to when the SBD binds an unfolded protein; there is also evidence that substrates do not need to be completely unfolded before interacting with an Hsp70 [83,84]. Therefore, the SBD of the P417S mutant might be altered in a way that reduces binding to this specific peptide but is better able to bind another class of substrates, such as those represented by FFLux. It is also possible that the greater acceleration of the ATPase cycle in P417L compromises its ability to capture and refold FFLux more than P417S.

The inability of yeast expressing either Ssa1 mutant to grow at 37°C suggests that there are essential Ssa1 functions *in vivo* that require wild type-like activity at elevated temperatures. Simply expressing more P417L or P417S Ssa1 fails to rescue this growth defect: These proteins are naturally upregulated when expressed under the control of their endogenous promoters, even at permissive temperatures. Activities that might be compromised include protein translocation, or the folding of another essential protein, which in turn cannot be overcome by the compromised chaperone activity evident at higher temperatures.

With significant changes in the ATP hydrolytic cycle and decreased interactions with peptide substrates at permissive and even lower temperatures, it is unknown why yeast expressing the P417L or P417S proteins remain viable and grow relatively well at the permissive temperature. This might be due to the observed upregulation of the mutant chaperones, or formally it could be that the mutant proteins—once stripped from the concentrated, chaperone-rich milieu in which they exist—exhibit more severe defects *in vitro* than *in vivo*. We did observe modestly slower growth, a delay in recovery from stationary phase, and more mild defects in ERAD at permissive temperatures (data not shown), which reinforces the notion that there is limited loss of function under conditions when cells are viable. Moreover, consistent with partial rescue of mutant Ssa1 activity that occurs *in vivo* upon upregulation, improved FFLux refolding was measured when higher amounts of chaperone were supplemented into the assay. It follows, then, that the reduced chaperone capacity of the mutants is seen as a cellular stress, and that the yeast correspondingly induce Ssa1 expression to maintain essential levels of chaperone activity. Together, these data emphasize the flexible nature of Hsp70 levels and activity that allows cells to tolerate a wide range of conditions. More generally, the ability of cells to tolerate a range of Hsp70 activity is highlighted by the effort to target Hsp70 for therapeutic treatments [85,86]. In these cases, it is desirable to modulate activity in a way that specifically affects cells with a high reliance on Hsp70 activity (e.g., cancer cells) without destroying essential Hsp70-dependent functions in “normal” cells [87–90]. Current efforts have been devoted to finding more selective Hsp70 inhibitors, and the allosteric coupling between NBD and SBD has proven to be an attractive target [56,83,91,92]. Greater knowledge of the unique effects of specific coupling residues in Hsp70, such as P417, will allow for more targeted approaches in drug design.

In sum, mutants such as the P417L and P417S proteins provide an important tool to define Hsp70 structure-activity relationships, and indicate how unique residues might be altered to better probe Hsp70 function in other settings. It is also now desirable to review the many phenomenon that have been observed in *ssa1-45* yeast in light of the observation that loss of

biological function may not be associated with a loss of protein structure but instead arises due to the biochemical defects associated with the P417 mutation.

Materials and Methods

Plasmid and yeast strain construction

The *SSA1* gene from plasmid p416 Ssa1 [65] was used as a template for site directed mutagenesis using the Quick Change Kit (Agilent), according to the manufacturer's instructions. After mutagenesis to change residue 417 to either a Leu (codon CTA) or Ser (codon TCA), the resulting plasmids were isolated and sequenced. Plasmids with the correct change were digested with SacI and PvuII to remove the *SSA1* gene, which was isolated and ligated into the SacI-SmaI site of vector pRS317, which contains the *LYS2* selectable marker. The resulting pRS317-Ssa1 plasmids were again sequenced and then used to transform yeast strain AJM28-A [93]. AJM28-A carries His-tagged Ssa1 under the control of the *GAL* promoter on a *URA3* marked plasmid. After transformation cells were grown on plates lacking lysine and containing glucose to select for the desired pRS317-Ssa1 construct and to repress expression from the *GAL* promoter. To eliminate the resident *URA3* plasmid, colonies from the transformation were twice selected and streaked on plates containing 5-Fluoroorotic Acid. The resulting yeast strains were then re-checked for growth on medium lacking uracil to ensure that the original plasmid was lost. The newly introduced plasmid was also reisolated from the cells and sequenced to ensure that the desired wild type or *ssa1* mutant gene was present.

Protein purification

Wild type and the mutant Ssa1 proteins were purified as previously described [40] from the indicated strains, which were constructed as described above. In brief, yeast expressing Ssa1 from the native promoter were grown to an optical density at 600 nm (OD_{600}) of 2–3 and harvested by centrifugation. The cells were washed in water, suspended in 20 mM HEPES pH 7.4, 50 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 5 mM β -mercaptoethanol, 1mM PMSF, 2 μ g/ml Leupeptin, 1 μ g/ml Pepstatin A, and disrupted by vigorous agitation on a Vortex mixer with glass beads. The cell lysate was clarified by centrifugation and then proteins were bound to a 15ml DEAE sepharose column (GE Healthcare). Proteins were eluted with a 50–500 mM NaCl gradient and peak Ssa1 fractions were loaded directly onto a 5 ml ATP-agarose column (Sigma). The column was washed with buffer and bound proteins were eluted with 2 mM ATP. Next, the eluted protein peak was loaded onto a 10 ml hydroxyapatite (Biorad) column equilibrated in 20 mM KPO_4 pH 7.0. Bound proteins were eluted with a 20–300 mM KPO_4 gradient, and the peak Ssa1-containing fractions were concentrated using an Amicon ultra spin column. The concentrated protein fraction was then loaded onto a 60 ml Sephacryl 200 size exclusion column run in 20 mM HEPES pH 7.0, 200 mM KCl, 5 mM $MgCl_2$. Finally, the purified Ssa1 fractions were dialyzed extensively against 10 mM HEPES pH 7.0, 50 mM NaCl, 10% glycerol, and snap-frozen and stored at $-80^\circ C$.

To determine the relative amount of residual nucleotide bound to each protein after purification we subjected a 10 μ M sample to precipitation with trichloroacetic acid and

centrifugation to remove the denatured protein. The supernatant was neutralized with NaOH and the absorbance at 260 nm was measured, as described [94]. A sample containing only buffer and lacking protein was subjected to the same procedure and was used as a blank. The absorbance values at 260 and 280 nm were compared to those obtained from a set of ADP standards of known concentration, and after adjustment for dilution, the amount of released nucleotide was calculated. The final nucleotide content of our protein preparations was estimated to be 58% for wild type Ssa1, 41% for P417L, and 51% for P417S on a mole/mole basis.

Ydj1 was expressed in *E. coli* strain BL21 and purified using DEAE and hydroxyapatite as described [59]. The Hlj1 J domain fused to GST was expressed and purified on glutathione agarose as described [46]. Fes1 was expressed as a GST fusion protein [95] and purified using glutathione agarose.

Circular dichroism spectroscopy

The CD spectra of wild type and mutant Ssa1 proteins were obtained in 10 mM HEPES, 50 mM NaCl, 10% glycerol at a 5 μ M protein concentration with a Olis DSM17 Circular Dichroism spectrometer using a 0.1 cm quartz cuvette. The temperature was controlled using a Peltier device. Spectra were measured from 260 nm to 200 nm with 1 nm steps. Baseline spectra (buffer absorbance) were subtracted and the spectra of the samples were converted to molar ellipticity per residue ($\text{deg} \times \text{cm}^2 \times \text{dmol}^{-1}$).

Thermal denaturation experiments

The stability of the purified Ssa1 proteins was assessed using thermal denaturation and monitored by SYPRO Orange (Invitrogen) binding, essentially as described [96]. Purified Ssa protein was diluted to a final concentration of 2 μ M in 10 mM HEPES, 50 mM NaCl, 10% glycerol and mixed with 5 $\times$ SYPRO Orange in a final volume of 50 μ l. The reactions were supplemented with either buffer, or a final concentration of 500 μ M ADP or ATP. Reactions were transferred to 96-well plates, which were sealed with optical plastic tape and read on an ABI 7900HT Q-PCR machine using ROX as a reporter. Reactions were initially incubated at 25°C for 2 min, then ramped up to 90°C at a 1% ramp rate (~1.5 degrees/min).

Assays to monitor ATP hydrolysis and nucleotide release

ATP hydrolysis was measured in 20 mM Tris-Cl pH 7.4, 50 mM KCl, 5 mM MgCl_2 , 1mM DTT in a 20 μ l volume at 25°C. Ssa1 was present at a final concentration of 1 μ M, and the co-chaperones were added at the indicated concentrations. After mixing, the reactions were incubated for 10 min at 25°C and the assay was started by the addition of [α - ^{32}P]-ATP (Perkin Elmer) into a final concentration of 50 μ M unlabeled ATP. Aliquots were removed into stop solution and the amount of ATP hydrolysis was determined by spotting samples on PEI cellulose TLC plates and then resolving ADP from ATP in a 0.5M LiCl-1M formic acid mixture. The plates were dried and exposed to a phosphorimager screen and imaged with a Typhoon FLA7000 scanner. The amount of ATP hydrolysis was determined with ImageJ software. Background activity of all reaction components except Ssa1 was subtracted and the ATP turnover rates were calculated from the initial linear time points.

To measure the NEF-dependent release of nucleotide, the purified Ssa1 proteins were incubated with [α - 32 P]-ATP to form a chaperone-nucleotide complex as described previously [51]. Due to differences in the inherent rates of ATP hydrolysis, the mixtures were incubated for 30 min to allow hydrolysis of ATP to ADP. A 15 μ l aliquot was mixed with either buffer or GST-Fes1 to a final concentration of 1 μ M and incubated at 30°C for 1 min. The mixture was then quickly loaded on a G-50 spin column and the protein was separated from released nucleotide by centrifugation. The amount of remaining bound nucleotide was quantified by scintillation counting.

Single turnover ATP hydrolysis was measured by forming protein-nucleotide complex as described for the nucleotide release assays above, and immediately freezing the purified complex in liquid nitrogen to preserve the unhydrolyzed bound ATP. The amount of ATP and ADP in each complex after isolation can be seen in Supplemental Figure 2. The assay was started by quickly thawing the complex and placing the reaction tube at 25°C. Ydj1 was present at a final concentration of 1 μ M and the ala-p5 peptide was present at a final concentration of 100 μ M. Aliquots were removed at the indicated time points and reaction products were resolved by TLC, as described for the steady state assays (see above). The amount of remaining ATP and the amount of ADP formed was determined at each time point and the data were fit to a single exponential decay using Graphpad Prism software.

Protease digestion assay

The indicated Ssa1 protein (10 μ g) in 20 μ l of 20 mM Tris pH 7.4, 50 mM KCl, 5 mM MgCl_2 with 5 mM ADP was incubated for 20 min on ice. Proteinase K (Sigma) at a final concentration of 20 μ g/ μ l was diluted 1/100 in assay buffer and 1 μ l of this solution was added to each reaction. Aliquots (6 μ l) were removed and then added to 3 μ l of ice-cold 100% trichloroacetic acid (TCA) and the mixture was centrifuged at 17,000g in a Sorvall Legend Micro 17R centrifuge for 10 min. After the supernatant was carefully removed, the precipitated protein was washed in ice-cold acetone, dried, and resuspended in SDS-PAGE sample buffer. The protein products were analyzed by SDS-PAGE and Coomassie blue staining.

Firefly luciferase refolding and peptide binding assays

Firefly luciferase (Sigma) at a final concentration of 80 nM in refolding buffer (20 mM Tris-Cl pH 7.4, 50 mM KCl, 5 mM MgCl_2) was denatured at 42°C for 20 min followed by an incubation on ice for and an additional 20 min. The denatured luciferase (12.5 μ l) was added to reaction tubes with 12.5 μ l refolding buffer, which included the indicated chaperones and co-chaperones for a final volume of 25 μ l. The concentrations of the proteins and other reagents were: 40 nM luciferase, 1 μ M Ssa1, 1 μ M Ydj1, 5 mM DTT, and 5 mM ATP. Next, the reactions were incubated at 25°C and 2 μ l samples were removed at various time points, mixed with 50 μ l of luciferase assay reagent (Promega), and luciferase activity was measured in a Monolight 2010 luminometer. The background activity of a reaction with no added chaperones was subtracted from each data point.

To measure the binding of Ssa1 proteins to a peptide substrate, an Hsp70 Fluorescence Polarization (FP) saturation assay was performed as outlined [57]. Briefly, FP assays were

conducted in 25 mM HEPES pH 7.2, 20 mM NaCl, 4 mM DTT, 20 μ M CaCl₂, 110 mM KOAc, 2 mM Mg(OAc)₂, and 0.01% NP40 and carried out in black 96-well microplates (Greiner Microtron Fluotrac 200) in a total reaction volume of 100 μ l. A stock of 10 μ M ala-p5-cy3b was prepared in DMSO and diluted with buffer. Increasing amounts of wild type Ssa1, P417L, or P417S (up to 500 nM) were incubated with 5 nM of ala-p5-cy3b. Next, the assay plate was incubated on a shaker at 4°C for the indicated times, and the FP values in mP were measured. The assay window was calculated as the difference between the FP value recorded for the bound fluorescent tracer and the FP value recorded for the free fluorescent tracer (defined as mP - mPf). Measurements were performed on a Molecular Devices SpectraMax Paradigm instrument (Molecular Devices, Sunnyvale, CA), and data were imported into SoftMaxPro6 and analyzed in GraphPad Prism 5.

Yeast cell growth and selection

Yeast were grown on standard yeast extract-peptone-dextrose (YPD) medium or on synthetic complete (SC) medium lacking the appropriate selective nutrient for strains carrying plasmid vectors. Plasmids were introduced into yeast by the lithium acetate method of transformation [97]. For spot assays, the cells were grown to saturation overnight in selective medium. A series of 5-fold serial dilutions were then made and spotted on selective growth medium and incubated at the indicated temperatures for 3 days.

Protein degradation and translocation assays

Wild type or mutant *ssa1* yeast strains were transformed with a plasmid expressing the HA tagged Ste6* protein and the degradation of this integral membrane ERAD substrate was analyzed by cycloheximide chase as described [98]. In brief, the cells were grown at 26°C to an OD₆₀₀ of 0.8–1.2, and the cultures were shifted to the assay temperature for 10 min and cycloheximide was added to a final concentration 100 μ g/ml. Aliquots of cells were removed at the indicated time points, total cell protein was isolated by TCA precipitation, as described above, and Ste6* levels were analyzed by SDS-PAGE and western blotting. Data were visualized on a Kodak 440CF image station and quantified with Kodak 1D software.

To monitor the translocation of ppa_f into the yeast ER, wild type or *ssa1* mutant yeast strains were transformed with a plasmid expressing an HA tagged version of yeast prepro α factor (ppa_f) that could not be glycosylated and thus processed beyond the ER [99]. The transformed cells were grown at 26°C to an OD₆₀₀ of 1.0, the cultures were split, and half of the culture was shifted to 37°C for 1 hr. An equal number of cells in each culture were collected and total protein was extracted by TCA precipitation (see above). The proteins were separated on 18% SDS-polyacrylamide gels and the presence of untranslocated ppa_f and the ER-resident, signal sequence processed paf was analyzed by western blotting and visualized as outlined above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ATP	adenosine triphosphate
ADP	adenosine diphosphate
NEF	nucleotide exchange factor
SBD	substrate binding domain
NBD	nucleotide binding domain
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum associated degradation
PMSF	phenylmethylsulfonyl fluoride
DTT	dithiothreitol
DMSO	dimethyl sulfoxide
TCA	trichloroacetic acid
TLC	thin layer chromatography
GST	glutathione <i>S</i> -transferase
WT	wild type

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Highlights

- *ssa1-45* (P417L) is a commonly used temperature sensitive Hsp70 allele in yeast
- Ssa1 P417L and another mutant, P417S, were purified and characterized
- The mutants are unregulated ATPases and are substrate binding defective
- Substrate binding domain integrity and interdomain coupling are altered
- P417 cellular defects do not correlate with temperature dependent loss of activity

Fig. 1. P417 resides in the substrate binding domain. After sequence alignment, the location of Pro 417 (P417) was mapped onto the structure of the *E. coli* protein DnaK using Protein Data bank file 2KHO. The NBD is shown in dark blue, the SBD β structure is in light blue, and the α helical lid is in gold. (A) Proline 417 is located in the substrate binding domain in the loop between the 2nd and 3rd β strands. (B) A magnification of the structure surrounding residue 417 and its relation to the SBD and the interdomain linker region (in red) is shown.

Fig. 2.

The steady state ATPase activity of the P417 mutant Ssa1 proteins is unregulated. (A) The time course of ATP hydrolysis of purified wild type Ssa1, P417L, or P417S was determined as described in the Materials and Methods. Data represent the means, $\pm$ S.E., for 3 independent experiments. Note that the differences between the two mutants in this data set were significant only at the 60 minute time point ($p = 0.02$), but we consistently saw slightly higher activity with the P417L protein (data not shown). (B) The ATPase activity of the indicated proteins was assayed with (1) Ssa1 alone, (2) upon addition of the peptide ala-p5

(at 125 μ M), or with the J domain-containing co-chaperones (3) Hlj1 or (4) Ydj1. The numbers over the bars reflect the fold increase in activity compared the unstimulated, intrinsic activity of each protein. The graph represents the means, $\pm$ S.E., for 4–6 measurements for each protein. * ($p= 0.02$)

Fig. 3.

The P417 Ssa1 mutant proteins exhibit higher rates of ATP hydrolysis in single turnover studies. Protein/nucleotide complexes were incubated at 25°C alone or with 1 μ M Ydj1 or 100 μ M ala-p5 peptide. Aliquots were removed every 20 seconds and the (a–c) % ATP remaining and (d–f) % ADP formed was determined. Each data set was fit to a single exponential decay to determine the rate of ATP hydrolysis. The graphs represent the disappearance of ATP of (A) Wild type Ssa1, (B) P417L, and (C) P417S, or the appearance of ADP for (D) Wild type Ssa1, (E) P417L, and (F) P417S. Circles represent reactions with the Ssa1 complexes alone, triangles with Ydj1, and squares with ala-p5.

Fig. 4.

Fes1 catalyzes the release of ADP from Ssa1. (A) The ATPase activity of the Ssa1 proteins (1 μ M) upon addition of increasing concentrations (0, 1, or 2 μ M) of the nucleotide exchange factor Fes1 was assessed. Activity in the absence of Fes1 was set to 1 and the bars reflect the fold increase upon addition of increasing amounts of Fes1. (B) The indicated Ssa1 proteins after incubation with radiolabeled ATP were collected by centrifugation through G-50 columns. Scintillation counting of the re-isolated Ssa1 proteins before (–) and after (+) incubation with Fes1 and re-purification on G-50 spin columns were used to quantitate the

relative amount of the bound radioactive nucleotide. The bars represent the means, $\pm$ S.E., of 4 independent measurements for each protein. Numbers above the bars indicate fold change in the amount of bound nucleotide relative to the control.

Fig. 5.

The P417L and P417S chaperones are peptide binding defective. (A) Increasing concentrations of the Ssa1 proteins were incubated with a fluorescently labeled peptide, cy3b-ala-p5, at a final concentration of 5 nM, and fluorescence polarization was measured at the indicated protein concentrations. The data were graphed and analyzed by Graphpad Prism software. mP refers to milli-polarization units (see Materials and Methods). The curves represent the average of two separate experiments for each protein with a total of 4 replicate measurements. (B) The proteins are stable over the time course of the assay.

Coomassie stained gel after SDS-PAGE showing the Ssa1 proteins: (1) No treatment, (2) after 24 hour incubation under the assay conditions, (3) supernatant from the 24 hour sample after centrifugation, and (4) pellet fraction of the 24 hour sample after centrifugation.

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Fig. 6. Reactivation of heat denatured firefly luciferase by Ssa1 and Ydj1. Firefly luciferase was inactivated at 42°C and then added to refolding reactions containing the indicated Ssa1 proteins as well as Ydj1. Aliquots were removed after 15, 30, and 60 min and luciferase activity was measured in a luminometer. (A) Percent of restored luciferase activity over a refolding time course. Ssa1 proteins were present at a final concentration of 1 μ M or 2 μ M, as indicated. Ydj1 was present at 1 μ M in all reactions. Open circles are wild type Ssa1, grey squares and grey triangles are P417S, and black squares and black triangles are P417L. The

graph represents the means, $\pm$ S.E., of 4–5 independent measurements at each time point. (B) The relative luciferase activity at the 60 min time point is shown. Refolding in the presence of 1 μ M wild type Ssa1 and 1 μ M Ydj1 was set to 100 and the other reactions were expressed as a percentage of this value.

Fig. 7.

Protease digestion of the Ssa1 proteins indicates that the substrate binding domains of P417 mutant chaperones exhibit protein folding defects. Purified wild type Ssa1 and the P417L and P417S mutant proteins were digested with Proteinase K in ADP at 30°C. Aliquots were removed at 0, 2, 5, or 10 min and analyzed by SDS-PAGE. The positions of full length Ssa1 (FL) and the free nucleotide binding domain (NBD) are indicated, as are molecular weight standards ($\times 10^{-3}$).

Fig. 8.

The growth of yeast expressing the P417L and P417S Ssa1 mutants is temperature sensitive. (A) Yeast expressing wild type Ssa1 or the P417L or P417S chaperones as the only copy of an Ssa protein were serially diluted 5-fold and spotted onto growth medium. The plates were incubated at 26°C, 30°C, 34°C or 37°C for 3 days. (B) Yeast cells expressing each Ssa1 variant were grown to log phase in liquid culture at 26°C and the cells were shifted to the indicated temperatures for 2 hr. An equal number of cells were harvested and the amount of Ssa1 protein was evaluated by western blotting. Wild type expression was set to 1, and the relative amounts of the mutant proteins are expressed as a fold increase relative to the wild type levels. The signal for wild type increased 2-fold from 26°C to 37°C. The values represent the means, $\pm$ S.E., for 3 separate yeast cultures.

Fig. 9.

Yeast expressing the Ssa1 p417 mutants exhibit protein degradation and translocation defects. (A) Cycloheximide chase analyses to detect the rate of Ste6* degradation were performed in yeast strains expressing wild type Ssa1 (white circles) or the P417L (black triangles) or P417S (grey squares) mutant chaperones as the only copy of Ssa in the cell. Cells were grown at 26°C and shifted to 37°C for 10 min prior to the start of the assay. Aliquots were removed at the indicated time points and Ste6* levels were evaluated by western blot. The graph displays the means, $\pm$ S.E., for 6 experiments. (B) A temperature

dependent translocation defect for yeast ppaf is evident in the Ssa1 mutant strains. An epitope-tagged copy of a yeast ppaf that could not mature beyond the ER was expressed in cells containing wild type Ssa1 or the P417L or P417S mutant chaperones. Cells were grown to log phase at 26°C and half of the culture shifted to 37°C for 1 hr. Cells were harvested and ppaf levels were evaluated by western blot. The position of the processed pro- α factor (paf), which resides in the ER, and the unprocessed pre-pro- α factor (ppaf), which is untranslocated, are indicated.

Table 1

Tmelt values for the Ssa1 proteins under different nucleotide conditions

	ATP ^a	ADP ^a
Wild type	50.0 +/- 0.4	50.8 +/- 0.4
P417L	49.2 +/- 0.5	49.8 +/- 0.6
P417S	51.1 +/- 0.6	51.5 +/- 0.7

^a ATP and ADP were added at a final concentration of 500 μ M^b Values represent the mean midpoint transition temperature in $^{\circ}$ C +/- SD for 4 independent measurements of each protein