Cumhuriyet Tıp Derg 2011; 33: 26-32
Cumhuriyet Med J 2011; 33: 26-32

Original research-Orijinal araştırma

Ssa1p function in the presence of nucleotide and Ssa1p oligomeric properties

Nükleotid varlığında Ssa1p işlevi ve Ssa1p oligomerik özellikleri

Yusuf Tutar

Department of Biochemistry (Assoc. Prof. Y. Tutar, PhD), Cumhuriyet University School of Medicine, TR-58140 Sivas

Abstract

Aim. To investigate *S. cerevisiae* cytosolic Hsp70-Ssa1 protein thermal unfolding in the presence of ATP and a substrate protein A7. **Method.** Plasmid preparation was done according to the standard protocols. pC210 was used to overexpress Ssa1p and Ssa1-A17V. Plasmids were linearized and integrated in *Pichiapastoris* (strain GS 115) genome. Transformants were selected by YPD/zeocin/sorbitol plates. Harvested cells were lysed by silica beads and centrifuged to homogenity. Lysate was purified by following chromatographic purification. Nucleotide free Ssa1 protein was obtained by using Sephadex G-50 column. Gel filtration experiments on binding and aggregation studies were performed by using Sephacryl-200 resin and one meter column. Fluorescence experiments were performed with Shimadzu RF 450 spectrofluorometer. **Results.** Both Ssa1p and Ssa1-A17V showed no significant difference in substrate protein binding. The binding to peptide reaches a maximum around 40 °C and then a sharp decrease was seen. In the absence of nucleotides, both proteins form monomeric species between 20-40ºC. Above oligomeric forms were observed. However, in the presence of 1 mM ATP oligomerization starts around 60ºC. Increase in temperature did not result in a second transition but a decrease in fluorescence was observed for both proteins. **Conclusion.** A7 and nucleotide ATP alters Ssa1p oligomeric properties and stability. This result is consistent with physiological conditions in a cell and explains how a cell survive under mild stress conditions through expressing Hsp70s.

Keywords: Hsp70, S. erevisiae, Ssa1p

Özet

Amaç. *S. cerevisiae* sitozolik Hsp70-Ssa1 proteinin ATP ve A7 substarat protein varlığında termal açılmasını araştırmak. **Yöntem.** Plasmid hazırlanması standart protokollere göre yapıldı pC210 Ssa1p ve Ssa1-A17V'yi yüksek ifade etmet için kullanıldı. Plasmidler çizgisel hale getirildi ve *Pichiapastoris* (zincir GS 115) genomuna eklendi. Transforme olmuş hücreler YPD/zeosin/sorbitol tabakaları kullanılarak seçildi. Elde edilmiş hücreler silika yardımıyla lize edildi ve homojenleştirmek için santrifuje edildi. Oluşan lizat kromotografik saflaştırmayı takiben saflaştırıldı. Nükleotidsiz Ssa1 proteini Sephadex G-50 kolonu kullanılarak elde edildi. Bağlamada ve yapışma çalışmalarındaki jel fitirasyon deneyleri Sephacryl-200 rezin ve 1 metre kolon kullanılarak uygulandı. Floresan deneyleri Shimadzu RF 450 spektrofotometre kullanılarak yapıldı. **Bulgular.** Her iki Ssa1p ve Ssa1-A17V substurat protein bağlamada belirgin fark göstermediler. Pepti'de bağlanma maksimuma 40 °C civarında ulaşmaktaydı ve sonrasında keskin görüldü. Nükleotidlerin yokluğundan, her iki proteinde 20-40ºC arasında monomerik türler oluşturmaktadır. 40ºC 'nin üzerinde oligomerik şekiller görüldü. Bununla beraber 1 mM ATP'nın varlığında oligomerizasyon 60ºC civarında başlar. Isının artması ikinci bir geçişle sonlanmaz fakat florasandaki bir düşüş iki protein içinde gözlemlenmişti. **Sonuç.** A7 ve nüleotid ATP Ssa1p oligomerik özelliklerini ve stabilitesini değiştirir. Bu sonuç bir hücredeki fizyolojik koşullarla uygunluk gösterir ve hafif stress koşullarında Hsp70'leri ifade ederek nasıl hayatta kaldığını açıklar.

Anahtar sözcükler: Hsp70, S. erevisiae, Ssa1p

Geliştarihi/Received: October 04, 2010; **Kabul tarihi/Accepted:** January 04, 2011

Corresponding addres:

Dr. Yusuf Tutar, Biyokimya Anabilim Dalı, Cumhuriyet Üniversitesi Tıp Fakültesi, TR-58140 Sivas. E-posta: ytutar@gmail.com

Introduction

Different stress factors affect cellular function through perturbing protein macromolecule native structure. Universally conserved Heat Shock Protein (Hsp) family help organisms to maintain protein native state and therefore protein function [1-3]. Heat shock proteins are also called chaperones due to their substrate protein folding activity. Heat shock protein family consists of Hsp40, Hsp60, Hsp70, Hsp90, Hsp100, small Hsp and nucleotide exchange factors. Hsp70 plays a key role in the mechanism and Hsp70 has redundant forms event in the same compartment of a cell [4, 5].

For example *S. cerevisiae* cytosol has Ssa1-4p and Ssb1-2. Hsp70 has two distinct type; constitutive and inductive form. Why Hsp70 family has different redundant forms is not elucidated yet? Hsp70 cochaperon Hsp40 has also different forms and it has been proposed that combination of different Hsp70-Hsp40 complexes serve for different cellular function [2, 5]. Hsp70 has two different domains (Figure 1). ATPase domain and substrate protein binding domain. ATPase domain hydrolyzes ATP and transmits the energy to substrate binding domain. This domain has a cavity and a lid that covers the cavity. The lid opens-closes on the cavity upon ATP hydrolyses and the cavity forms an isolated space for substrate protein folding. The cavity is hydrophobic and help non native substrate proteins to reach their native state [2]. It should be remembered that a protein reach its native state mainly by hydrophobic interactions.

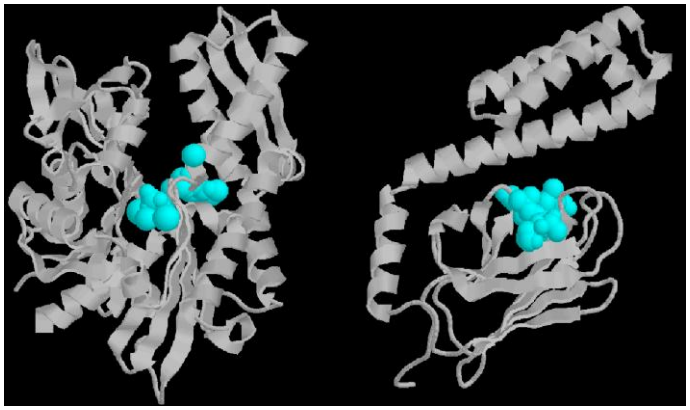

Figure 1. Left, ATPase domain of Hsp70 (PDB ID: 1S3X). Nucleotide was displayed in spacefill. Right, Substrate binding domain of Hsp70 (PDB ID: 1DKZ). Substrate peptide (asn-arg-leu-leu-leu-thr-gly) was displayed in spacefill. Figures were drawn with Rasmol software.

Stress may cause a protein to expose its hydrophobic regions and this increases surface area. Increased surface area increases protein-protein interaction since water-hydrophobic interactions drives exposed non native protein to find counter hydrophobic regions. This hydrophobic regions form aggregates. Aggregation cause a decrease in the total protein concentration and function. Loss of certain protein function may cause severe problems if the protein plays an important role. For example S. cerevisiae transcription termination factor protein, Sup35, may form aggregates and aggregates form prion, [PSI^{+}]. This functional failure causes perturbation of translation [6, 7]. Hsp70 coordinates and cooperates with several different proteins. Nucleotide exchange factors, Hsp40s and Hsp100 are among the key ones. Upon ATP hydrolyses, Hsp70 uses the energy to close the substrate binding domain lid to process substrate protein folding. However, ADP must be removed from Hsp70, so that a new cycle can be started. Nucleotide exchange factors

remove ADP nucleotide and regenerate the system [2]. Hsp40 proteins display a variety of functions with Hsp70s. Some of them behave as chaperone by hydrolyzing ATP. Hsp40s essential function is helping Hsp70s through submitting substrate molecules into Hsp70s substrate binding domain (Figure 2). This process facilitates Hsp70 function to process substrate proteins i.e. proper orientation of enzyme-substrate complex [2].

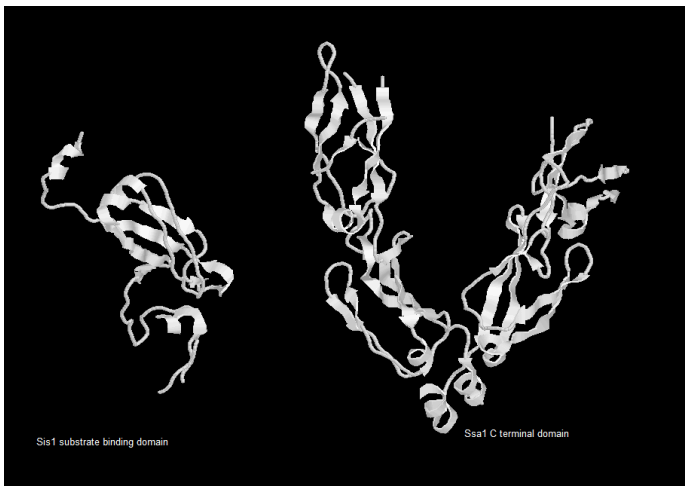

Figure 2. *S. cerevisiae* Hsp40 protein Sis1 and Ssa1 protein interaction (PDB ID: 2B26). Hsp40 interacts with Ssa1 ATPase domain. ATPase domains have conservative motif "EEVD" for this interaction. This interaction is necessary for Hsp70-Hsp40 communication. Figure was drawn with Rasmol software.

Hsp100 protein has unique structure to distangle aggregated substrate proteins. Hsp100 consists of dimeric structure. Each dimer has six sub-domains and all together the structure behave like a blade (Figure 3). The blade chops off a substrate protein among aggregates. According to the accepted model in the literature Hsp100 process the substrate by passing it from its central hole. During this process the substrate align itself. Then Hsp70-Hsp40 receive the substrate from Hsp100 to further process and eventually substrate reaches its native state [8, 9].

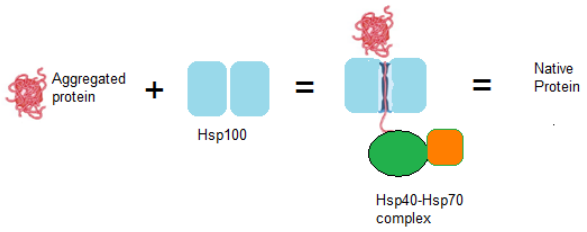

Figure 3. Hsp100 dissolves aggregates and grasp a peptide among them. The peptide pass from the hole and at the end of the hole Hsp70-Hsp40 complex further process the peptide to reach its native folded state.

Protein structure was inevitably affected by extreme conditions i.e. rise in temperature. Hsp70 thermal stability and oligomerization properties at different nucleotide conditions must be examined since Hsp70 rescues substrate proteins from non native states at these adverse conditions. This work investigates S. cerevisiae cytosolic Hsp70-Ssa1 protein thermal unfolding in the presence of ATP and a substrate protein A7 along with its substrate binding mutant form A17V. This mutant is placed in ATPase domain and accelerates substrate processing through accelerating ATP hydrolyses rate.

Materials and Methods

Yeast Strains, Plasmids and Growth Conditions

Plasmid preparation explained elsewhere [10, 11]. pC210 was used to overexpress Ssa1p and Ssa1-A17V. Plasmids were linearized and integrated in *Pichiapastoris* (strain GS 115) genome. Transformants were selected by YPD/zeocin/sorbitol plates. Minimal dextrose medium was used and Hsp70 induction was made by 0.5% methanol [10, 11].

Protein purification, peptide synthesis and peptide binding /aggregation

Harvested cells were lysed by silica beads and centrifuged to homogenity. Lysate was purified by following three step chromatographic purification; DEAE-Sephacel, butyl-sepharose and gel filtration according to Wegele et al. [12]. Molecular concentrations were determined by spectrophotometry, using extinction coefficients 35,700 and 15,400 M-1 cm-1 at 280 and 259 nm for Ssa1 and nucleotides (ATP and ADP) respectively. ATP-Na2 (purity ≥ 99%) was purchased from Sigma-Aldrich. ADP (purity ≥ 95%, Sigma-Aldrich) was further purified by Mono-Q column (GE Healthcare). Nucleotide free Ssa1 protein was obtained by using Sephadex G-50 column. Purified protein was diluted in Buffer A (25 mMHepes, 5mM MgCl2, 50 mMKCl, pH 7.4). Peptide A7 (RRLIEDAETAARG) (catalog number A7433) was obtained from Sigma. Gel filtration experiments on binding and aggregation studies were performed by using Sephacryl-200 resin and one meter column (GE Healthcare).

Fluorescence experiments

Fluorescence experiments were performed with Shimadzu RF 450 spectrofluorometer. The excitation wavelength was set at 295 nm for intrinsic fluorescence. Excitation and emission wavelengths set to 5 nm. Fluorescence signal was blanked with mock experiments. A thermocycler was employed for protein incubation. Protein samples were heated from ambient temperature to the indicated temperatures for 3 min [6, 7].

Results

Effect of temperature on the binding of A7 peptide t"o Ssa1p and Ssa1-A17V.

A mixture of Ssa1p or Ssa1-A17V (5 μM) and A7 (20 μM) were incubated for 10 minutes at indicated concentrations and run on Sephacryl-200 (GE Healthcare) at room temperature. Percent bound peptide was calculated by comparison of A7-Ssa1 and Ssa1 peaks (Figure 4). Both Ssa1p and Ssa1-A17V showed no significant difference in substrate protein binding. The binding to peptide reaches a maximum around 40 °C and then a sharp decrease afterwards [6, 7, 13].

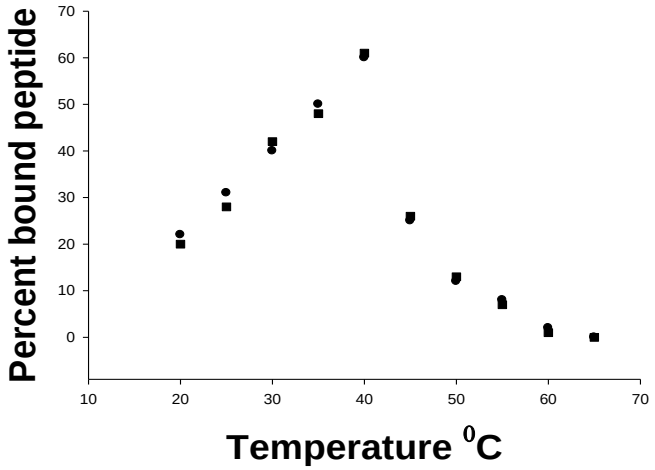

Figure 4. Binding of substrate protein A7 to Ssa1p (●) and Ssa1-A17V (■) mutant in the presence of 1 mM ATP.

Aggregation properties of Ssa1p

Thermal oligomerization of Ssa1p and Ssa1-A17V (5 µM) followed by gel filtration with (●,■) and without (○, □) 1 mM ATP nucleotide (Figure 5). In the absence of nucleotides, both proteins form monomeric species between 20-40ºC. Above 40ºC oligomeric forms were observed. However, in the presence of 1 mM ATP oligomerization starts around 60ºC [6, 7, 13].

Thermal stability of Ssa1p followed by fluorescence

Increase in temperature did not result in a second transition but a decrease in fluorescence was observed for both proteins (for the sake of clarity Ssa1-A17V data was not given). In the absence of nucleotide the transition was observed around 40°C but in the presence of ATP and A7 peptide the transition shift to 60°C (Figure 6) [6, 7, 13].

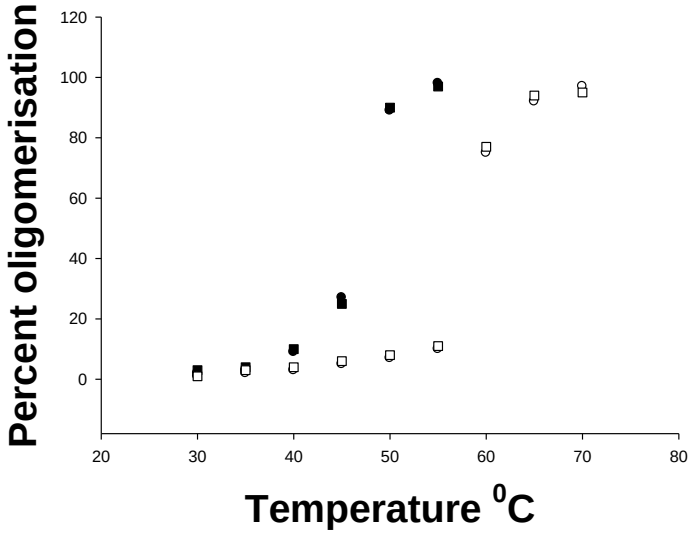

Figure 5. Thermal oligomerization of Ssa1p and Ssa1-A17V (10 µM) with (●,■) and without (○, □) 1 mM ATP nucleotide.

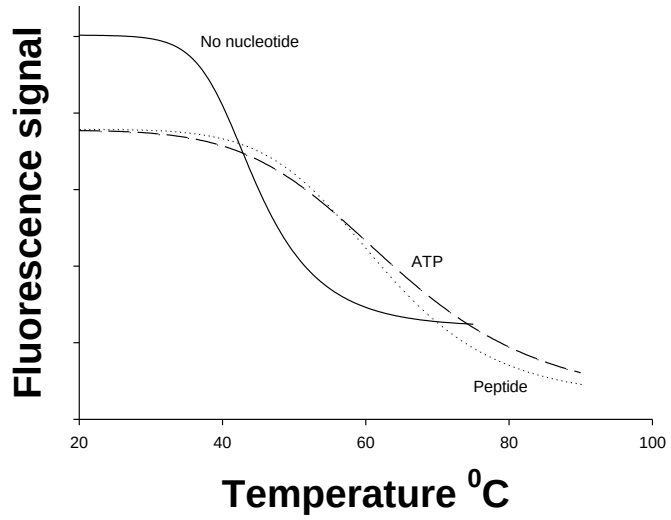

Figure 6. Thermal stability of Ssa1p and Ssa1-A17V (5 µM) with no nucleotide, with 1 mM ATP and with peptide A7 (5 µM).

Discussion

Environmental conditions affect cell and typical temperature stress primarily perturbs protein structure. Hsp70s guide non native proteins to reach their native state under these conditions but they themselves are exposed to the same stress [2].

Therefore, effect of temperature on the substrate binding to Ssa1p at different temperatures was investigated and results presented in Figure 4. Figure 4 indicates that denatured substrate protein binds Ssa1p even at 50ºC. Ssa1p thermal stability reaches a maximum around 40ºC. This stability helps substrate proteins to bypass minor stress affects through heat shock proteins. Ssa1-A17V mutant was employed to see any changes in function since Ssa1-A17V hydrolyzes ATP better than Ssa1p. Increase in hydrolysis rate may enhance peptide binding. However, Ssa1-A17V showed similar characteristics indicating that denatured substrate protein requires similar conditions. This mutant may affect kinetics of substrate protein to reach native state. Ssa1p aggregates and forms oligomeric structures as shown by Figure 5. In the absence of nucleotide, Ssa1p and Ssa1-A17V are monomeric up to 40ºC. Then oligomerization starts around 55ºC the proteins become oligomeric completely. The oligomerization is irreversible and in the presence of ATP oligomerization temperatures shift to higher temperatures by ca. 10ºC. Oligomers formed at all protein concentrations tested.

When thermal stability of the proteins was followed by fluorescence (Figure 6) a cooperative transition around 40ºC was observed in the absence of nucleotide. Addition of nucleotide and nucleotide plus peptide shifted this transition around 60ºC. This transition of Ssa1p and Ssa1-A17V corresponds to a loss in tertiary structure.

In conclusion A7 and nucleotide ATP alters Ssa1p oligomeric properties and stability. This result is consistent with physiological conditions in a cell and explains how a cell survive under mild stress conditions through expressing Hsp70s.

Acknowledgements

This work was funded partly by the Turkish Planning Organization (DPTK.120220-2006) and through a seed grant from the Turkish National Academy of Sciences (TUBA-GEBIP).

References

1. Tutar Y. Therapeutic use of heat shock proteins and essential factors in prognosis, diagnosis and treatment of neurodegenerative and metabolic diseases.Curr Pharm Biotechnol 2010; 11: 138.
2. Tutar L, Tutar Y. Heat shock proteins; an overview. Curr Pharm Biotechnol 2010; 11: 216-22.
3. Tutar Y. Prelude; cellular mechanics. Protein PeptLett 2009; 16: 570.
4. Tutar L, Tutar Y. Ydj1 but not Sis1 stabilizes Hsp70 protein under prolonged stress in vitro. Biopolymers 2008; 89: 171-4.
5. Tutar Y. Therapeutic use of heat shock protein 70. Recent Pat DNA Gene Seq 2007; 1: 125-7.
6. Tutar Y. Heat shock proteins, substrate specificity and modulation of function. Protein Pept Let 2006; 13: 699-705.
7. Tutar Y. Key residues involved in Hsp70 regulatory activity and affect of co-chaperones on mechanism of action. Protein PeptLett 2006; 13: 693-8.
8. Masison DC, Kirkland PA, Sharma D. Influence of Hsp70s and their regulators on yeast prion propagation. Prion 2009; 3: 65-73.
9. Reidy M, Masison DC. Sti1 regulation of Hsp70 and Hsp90 is critical for curing of Saccharomyces cerevisiae [PSI+] prions by Hsp104.Mol Cell Biol 2010; 30: 3542-52.
10. Tutar Y, Song Y, Masison DC. Primate chaperones Hsc70 (constitutive) and Hsp70 (induced) differ functionally in supporting growth and prion propagation in Saccharomyces cerevisiae. Genetics 2006; 172: 851-61.

11. Song Y, Wu YX, Jung G, Tutar Y, Eisenberg E, Greene LE, Masison DC. Role for Hsp70 chaperone in Saccharomyces cerevisiae prion seed replication. Eukaryot Cell 2005; 4: 289-97.
12. Wegele H, Haslbeck M, Buchner J. Recombinant expression and purification of Ssa1p (Hsp70) from Saccharomyces cerevisiae using Pichiapastoris. J Chromatogr B AnalytTechnol Biomed Life Sci 2003; 786: 109-15.
13. Chirico WJ, Markey ML, Fink AL. Conformational changes of an Hsp70 molecular chaperone induced by nucleotides, polypeptides, and N-ethylmaleimide. Biochemistry 1998; 37: 13862-70.