ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

STUDYING LINKER INDUCED EFFECTS TO THE ATPASE DOMAIN OF DNAK

M.Sc. THESIS

Ani KIÇİK

Department of Advance Technologies

Molecular Biology & Genetics and Biotechnology Programme

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

HSP70'LERİN ATPAZ PARÇASINDAKİ MOLEKÜLER AKTİFLEŞME MEKANİSMASININ ARAŞTIRILMASI

YÜKSEK LİSANS TEZİ

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Ani Kıçik, a **M.Sc.** student of ITU **Institute of** Science and Technology student ID 52101077, successfully defended the **thesis** entitled "**STUDYING LINKER INDUCED EFFECTS TO THE ATPASE DOMAIN OF DNAK**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family,

FOREWORD

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Molecular Biologist

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ABBREVIATIONS

ADP : Adenosine diphosphate
Amp : Ampicillin
APS : Ammonium persulfate
ATP : Adenosine triphosphate
ATPase : Adenosine triphosphatease
Bag : Bcl2-associated athanogene
CBB : Coomassie Brilliant Blue
CD : Circular Dichroism
DEAE : Diethylaminoethyl
DNA : Deoxyribonucleic acid
dNTP : Deoxyribonucleotide triphosphate
DTT : Dithiothreitol
E.coli : *Escherichia coli*
EDTA : Ethylenediaminetetraacetic acid
ER : Endoplasmic reticulum
FPLC : Fast protein liquid chromatography
Hsc : Heat shock cognate
HSE : Heat shock element
HSF 1 : Heat shock factor 1
Hsp : Heat shock protein
IPTG : Isopropyl β-D-1-thiogalactopyranoside
LDH : Lactate dehydrogenase
NADH : Nicotinamide adenine dinucleotide
NBD : Nucleotide binding domain
NEF : Nucleotide exchange factor
NMR : Nuclear Magnetic Resonance
PCR : Polymerase chain reaction
PDB : Protein Data Bank
PEP : Phosphoenolpyruvate
PK : Pyruvate kinase
PMSF : Phenylmethanesulfonylfluoride
rpoH : RNA polymerase, sigma 32 (sigma H)
SBD : Substrate binding domain
SDS : Sodium dodecyl sulfate
TEMED : Tetramethylethylenediamine
Tm : Melting temperature
UV : Ultraviolet
Wt : Wild-type

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STUDYING LINKER INDUCED EFFECTS TO THE ATPASE DOMAIN OF DNAK

SUMMARY

Hsp70 is a molecular chaperone that plays role in a variety of cellular activities, such as folding of proteins, prevent aggregation of proteins and membrane translocation. Hsp70 consist of two main domains; ATPase binding domain and substrate binding domain, which are connected by a highly conserved hydrophobic linker. When Hsp70 binds its substrate, an allosteric communication occurs between its ATP-binding and substrate-binding domains. Up till now, this allosteric communication between two domains of Hsp70 was tried to understand with several studies. Now, it is known that linker region acts as a molecular switch between two domains and thus, it plays an important role in the signal transducing mechanism. However, because of lacking knowledge about the structure and the mocular details of this linker region signal transducing mechanism are not understood completely. Previous studies using *Echerichia coli* Hsp70 homolog, DnaK showed that the construct containing the entire linker, DnaK(1-392), mimics the substrate-stimulated state and leading to an enhanced ATPase rate compared to the construct lacking the conserved linker region, DnaK(1-388) (Swain et al. 2007). In addition, studies of Swain et al. also demonstrated that the DnaK(1-392) and peptide-bound full-lenght DnaK show pH-dependent ATPase activity profile.

In this study, we aimed to understand allosteric mechanism underlying the linker binding effects to the ATPase domain by pinpointing the critical residues that are present in DnaK(1-388). According to our hypothesis, there might be a network among His226, Asp85, Thr225 and Arg71 residues that are near to active site and may have critical roles in the ATPase allostery. In this regard, mutagenesis was performed on these residues and pH-dependent ATPase assays were done to understand the roles of these residues in the pH-dependent ATPase activity. When pH-activity profiles of Thr225 and His226 replacements were compared to that of the ATPase constructs, we found that mutations of these sites did not alter the ATPase mechanism and we revealed that the activity mechanism of the ATPase domain is different for linkerless version, DnaK(1-388), as compared with DnaK(1-392). On the other hand, we found that replacement of Asp85 and Arg71 cause 16 fold increase in the ATPase rate indicating these residues have repressive effects on the modulation of ATPase activity in the linkerless version, DnaK(1-388). In this study, His295 was also investigated for its possible effect on the pH-dependent ATPase activity. We observed similar shift in the pH activity profiles of H295D and D85E mutantsas observed in the DnaK(1-392) may indicating these mutations can stimulate ATPase to a lesser extend than DnaK(1-392). In addition, to understand the structural effects of mutations we also did native gel electrophoresis and observed similar oligomerizations for D85E and H295D suggesting that linker binding cleft is exposed in the DnaK(1-388) in ATP-bound state and interact with negative residues.

HSP70'LERİN ATPAZ PARÇASINDAKİ MOLEKÜLER AKTİFLEŞME MEKANİSMASININ ARAŞTIRILMASI

ÖZET

Isı şok proteinleri familyasının bir üyesi olan Hsp70, evrimsel olarak tüm türler arasında yüksek derecede korunmuş hücre içerisinde birçok önemli görevi olan bir moleküler şaperondur. Hsp70'in sentezi, ısı şok protein familyasının bir üyesi olması gereği yüksek sıcaklık, oksidatif stres ve dehidrasyon gibi hücresel stres koşullarında yüksek derecede artar. Bu proteinlerin, stres koşullarında olduğu kadar normal hücresel koşullarda da kritik görevleri vardır. Hsp70'in başlıca görevleri olarak; yeni sentezlenmiş proteinlerin üç boyutlu yapılarının kazanılması, protein kümeleşmelerinin engellenmesi, hasar görmüş proteinlerin yıkımı, membranlar arasında protein translokasyonlarının sağlanması, regülatör proteinlerin aktivitelerinin kontrol edilmesi ve antijenlerin immun sisteme tanıtılması sıralanabilir.Hsp70'in hücre içerisinde üstlendiği bu önemli görevler, onu hücre homeostasisinde önemli bir rol edinmesini sağlar ki bu durum bu proteinin evrimsel olarak neden bu derece korunmuş olmasını açıklamaya yeter.Hsp70'in hücre içerisindeki bu önemli rolü, bu proteinlerin düzensiz çalışmaya başlaması durumunda birçok immunolojik ve nörodejeneratif hastalıklarla ilişkili hale gelmesine sebep olur.

Hsp70, N-terminalinde ATPaz bölgesi ve C-terminalinde substrat bağlanma bölgesi olmak üzere iki ana bölgeden oluşur.Bu iki bölge, evrimsel olarak korunmuş hidrofobik bir bağlaç bölgesi ile bağlıdır.ADP bağlı haldeyken iki ayrı proteinmiş gibi bağımsız davranan bu iki bölge, ATP bağlanması ile birbirleriyle iletişime geçerek konformasyonal değişiklikler sonucunda, birbirlerine sıkı bir şekilde bağlı hale gelirler.Bu süreç içerisinde, ATPaz bölgesine ATP bağlanması bu bölgeyi daha stabil hale getirirken, substratın bağlanma affinitesinin düşmesine sebep olur. Diğer yandan, substratın bağlanması ATP'nin ADP'ye hidrolizini tetikler. Bu durum ise substratın, substrat bağlanma bölgesinde saklı halde kalmasını sağlayarak katlanma sürecinin gerçekleşmesine neden olur. Bu iki yönlü allosterik etkileşim mekanizmasının temelinde moleküler anahtar görevi gören bağlaç bölgesi bulunmaktadır. Fakat bu bölgenin yapısı ve görevinin moleküler detayları tam olarak bilinmediğinden bu iki bölge arasında gerçekleşen sinyal iletim yolu tam olarak aydınlatılamamıştır.

Hsp70'in ATPaz bölgesine ait çeşitli ligand bağlı hallerde (ATP, ATP analoğu, ADP ve ko-şaperon bağlı) birçok kristalografik çalışma mevcuttur. Bu kristalografik yapılara göre ATPaz bölgesi iki lobdan oluşmaktadır (I ve II), her bir lob ise iki ayrı alt-bölge içermektedir (IA-IB ve IIA-IIB).Bunun yanında substrat bağlanma bölgesine ait de peptid bağlı veya olmayan hallerde çeşitli kristalografik ve NMR çalışmaları mevcuttur. Bunların dışında, Hsp70'in iki bölgesinin de bir arada bulunduğu tüm-dizimi içeren kristal yapılar mevcuttur fakat tüm-dizi Hsp70'i içeren farklı nukleotid ve substrat bağlanmış halleri ile elde edilmiş bir yapısal model mevcut değildir. Böyle bir yapısal modelin olmaması bu iki bölge arasındaki allosterik sinyal mekanizmasının anlaşılmasını zorlaştırmaktadır. Bugüne kadar, mevcut olan kristal ve NMR

yapılarından yola çıkarak birçok protein mühendisliği çalışması yapılmıştır. Bu çalışmalar, ATPaz bölgesindeki ve substrat bağlama bölgesindeki kritik öneme sahip birçok aminoasitin aydınlatılmasını sağlamıştır. Bu bölgelerdeki protein mühendisliği çalışmalarına ek olarak bağlaç bölgesinde yapılan çalışmalar ile bağlaç bölgesinin iki bölge arasındaki sinyal iletim mekanizmasında temel bir görevi olduğu anlaşılmıştır (Laufen ve diğ.,1999; Vogel ve diğ. 2006a; Kumar ve diğ. 2011). Bu çalışmalarda genel olarak DnaK'in 9 amino asitlik (^{383}GDVKDVLLL^{392}) bağlaç bölgesinde nokta mutasyonları oluşturulmuş ve yapılan mutasyon analizleri ile DnaK'in bağlaç bölgesindeki ^{389}(VLLL)^{392} dizisininin substrat stimulasyonu sayesindeki ATPaz aktivitisini önemli derecede etkilediği gözlenmiştir. Bağlaç bölgesindeki bu amino asitlerin substrat stimulasyonlu ATPaz aktivitesindeki rolleri, bağlaç bölgesinin iki bölge arasında sinyal iletiminde temel bir görevi olduğunu ortaya çıkarmaktadır. Ayrıca bu mutasyon çalışmaları dışında Dinler ve diğ. (2007)'nin bağlaç bölgesi üzerindeki çalışmaları da bu bölgenin sinyal iletiminde önemli bir rolü olduğunu göstermiştir. Bu çalışmalarda, DnaK'in ATPaz bölgesi dizilimlerinden bağlaç bölgesindeki ^{389}(VLLL)^{392} dizisini içermeyen DnaK(1-388) ve ^{389}(VLLL)^{392} dizisini içeren DnaK(1-392) kullanılmıştır. Çalışmalar sonucunda, DnaK(1-392)'in peptid bağlı haldeki tüm-dizi DnaK'ye benzer pH'a bağımlı bir ATPaz aktivite profili gösterdiği gözlenmiştir. Bunun yanında, bu çalışmada DnaK(1-388)'in peptid bağlı olmayan haldeki tüm-dizi DnaK'e benzer ATPaz aktivite profili gösterdiği gözlenmiştir. Bugüne kadar yapılan tüm çalışmalar ile Hsp70'in iki bölgesi arasındaki sinyal iletim mekanizmasında kritik role sahip bazı amino asitler belirlenmiş olsa bile bu iletişim mekanizmasının moleküler temelleri tam olarak anlaşılamamıştır.

Bu tez çalışmasında, yukarıda anlatılan daha önceki çalışmalar ışığında Hsp70'in iki bölge arasındaki sinyal iletim mekanizmasının moleküler temellerinin aydınlanmasını sağlamaya yönelik daha derin bir bakış açısı kazanılması amaç edinilmiştir. Bu bakımdan, DnaK(1-388) kullanılarak ATPaz allosterik mekanizmasında kritik öneme sahip olduğu düşünülen birçok amino asit ile mutasyon analizleri yapılmıştır. Bu mutasyon analizleri için seçilen residüler, ATPaz bölgesinin nükleotid bağlanma bölgesine yakın His226, Thr225, Asp85, Arg71 ve bu bölgeden biraz uzak olan His295'dir. Kurulan hipoteze göre, ATPaz bölgesinin IIA ve IIB alt-bölgelerini birbirine bağlayan esnek bir döngü üzerinde olan His226 amino asidi, bağlaç bölgesinin ATPaz bölgesinin IA ve IIA alt-bölgelerinin birleştiği alt kısmına bağlanması sonucunda konum değiştirip ATPaz bölgesinin IB alt-bölgesinde yani karşı lobda bulunan Asp85 ile hidrojen bağı kurmaktadır. Buna ek olarak, oluşan bu yapısal değişiklikler Thr225'in de ATPaz bölgesinin IB alt-bölgesindeki Arg71 ile hidrojen bağı kurmasına neden olur. Bu sayede ATPaz bölgesinin karşılıklı iki lobu arasında bir ağ oluşur. Bu ağın ancak ATPaz bölgesinin bağlaç ile etkileşmesi sonucunda veya substrat bağlanması sonucunda oluştuğu düşünülmektedir. Sonuç olarak bu ağ içerisindeki amino asitlerin ATPaz allosterik mekanizmasında önemli rolleri olduğu düşünülmüştür. Çalışma kapsamında, bu amino asitlerin rollerini anlamak amacıyla, nokta mutasyonları yapılmış ve mutasyonların etkilerini görmek için ATPaz aktivite ölçümleri yapılmıştır. Bu ölçümlerin hepsi pH 5.5 ile 8.5 aralığında yapılarak amino asitlerin literatürde gösterilmiş olan pH'a bağımlı ATPaz aktivitesine etkilerinin olup olmadığı anlaşılmaya çalışılmıştır.Bu bakımdan, ATPaz bölgesindeki pH'a duyarlı ikinci histidin amino asidi olan His295 residüsünün de seçilmesinin sebebi bu residünün pH bağımlı ATPaz aktivisinin bir kaynağı olabileceğinin düşünülmesidir. Nokta mutasyonları, genel olarak amino asitlerin yük farklılıkları veya boyut farklılıkları esas alınarak, onların aktivite üzerindeki etkilerini gösterecek şekilde dizayn edilmiştir. Bu bakımdan, 226 pozisyonundaki histidin amino asidi, alanin ve fenialanin amino asitleri

ile değişikliğe uğratılmıştır. Alanin amino asidi ile mutasyon yaratılmasının amacı bu amino asidin nötr olmasından dolayı, nötralizasyon sağlanarak tuz köprüsü kurulmasını engellemektir. Buna ek olarak, fenialanin amino asidinin seçilmesinin amacı, fenilalaninin histidin ile benzer boyutta olmasına rağmen nötr olmasından dolayı yine nötralizasyon sağlanarak tuz köprüsü kuramamasıdır. Bunların yanında, Thr225, Arg71 ve Asp85 residüleri de yine aynı şekilde nötralizasyon sağlanarak tuz köprüleri kurmaları engellensin diye alanin amino asidi ile mutasyona uğratılmıştır. Asp85 ayrıca glutamik asit ile de mutasyona uğratılmıştır. Glutamik asidin tuz köprüsü kurma özelliği vardır ancak aspartik aside göre daha uzun olmasından dolayı histidin ile oluşturduğu düşünülen tuz köprüsünü kuramayacağı ön görülmektedir. His295 ise eksi yüklü aspartik asit ile mutasyona uğratılarak yük farkından kaynaklı bir etkinin gözlenmesi amaçlanmıştır. Oluşturulan mutasyonların hepsi "site-directed mutagenesis" tekniği kullanılarak yapılmıştır. Daha sonra mutasyonların varlığı sekans analizi ile doğrulanmıştır. Bu nokta mutasyonlarını içeren mutant proteinlerin üretimi DnaK taşımayan ($DnaK^-$) BB1553 *E.coli* hücre serilerinde gerçekleştirilmiştir. Protein saflaştırılmaları tamamlandıktan sonra, pH 5.5 ile 8.5 aralığında ATPaz aktivite ölçümleri yapılmıştır.

Deneysel sonuçlarda, D85A ve R71A mutantlarının ATPaz aktivitelerinde yabanıl tip DnaK(1-388)'e kıyasla yüksek oranda artışa sebep oldukları gözlenmiştir. Bu iki mutant, ölçülen tüm pH değerleri için yabanıl tipe göre yaklaşık olarak 16 katlık bir artış göstermiştir. Bu sonuçlar, Asp85 ve Arg71 residülerinin ATPaz aktivitesinin düzenlenmesinde baskılayıcı etkilerinin olduğunu göstermektedir. Bunun yanında, H226A, H226F ve T225A mutantlarınınn pH aktivite profillerinin yabanıl tip DnaK(1-388)'e benzer olması bu mutasyonların ATPaz mekanizmasında bir etkilerinin olmadığını düşündürtmüştür. Bu mutantların pH ATPaz aktivite profillerinde DnaK(1-388)'e benzer olarak pH 6'da pik vermiş olmaları, pH 6 noktasında titre edilebilen residülerin olduğunu işaret etmektedir. İlginç olarak, pH 6'da DnaK(1-392)'nin pik vermiyor olması ve bunun yerine pH 7.6'da pik veriyor olması, bağlaç bölgesinin varlığında ve yokluğunda ATPaz mekanizmasının farklılık gösterdiğini işaret etmektedir. Bunların dışında, D85E ve H295D mutantları yabanıl tipten farklı bir pH aktivite profili sergilemiş ve DnaK(1-392)'ye benzer olarak pH 7.5 civarında bir aktivite artışı göstermişlerdir. Bu sonuçlar, bu mutantların belki de DnaK(1-392)'ye benzer bir konformasyon gösterip, DnaK(1-392)'den kıyasla daha az da olsa ATPaz aktivitesinin stimülasyonunda etkilerinin olduğunu düşündürtmüştür. ATPaz aktivite deneyleri dışında mutasyonların neden olduğu ikincil yapısal değişiklikleri gözlemlemek için yapılan denatüre edici olmayan (native) jellerde D85E ve H295D mutantlarının ATP varlığında benzer oligomerizasyon göstermeleri DnaK(1-388)'de bağlaç olmamasından dolayı IA ve IIA alt bölgeler arasındaki hidrofobik bölgesinin ATP-bağlı haldeyken açık olması sayesinde eksi yüklü residüler ile etkileşime girerek oligomerizasyona yol açtığını düşündürtmüştür.

1. INTRODUCTION

1.1 Heat Shock Proteins

Heat shock proteins were first discovered in 1962 during a study based on the investigations of temperature effect on *Drosophilia melanogaster* larvae (Ritossa, F., 1962). It was observed that heat treatment of *Drosophila* larvae caused dramatic changes in the puffing pattern of polytene chromosomes in salivary glands, and later it was shown that this chromosomal alteration results in very active gene transcription of a small set of proteins. Therefore, this special class of proteins was described as heat shock proteins (HSPs) whose expressions were induced by heat shock. However, it is now known that their expressions are induced by not only heat shock but also by other stressors such as altered pH, oxidative stress, chemical perturbations and ethanol.

Heat shock proteins are an evolutionarily highly conserved family of proteins from bacteria to human. They play critical roles in order to protect the essential cell components against heat damage or other stress conditions by preventing the misfolding and aggregation of proteins. Because of these functions they are described as the primary system for intracellular self-defense. Heat shock proteins are also referred as “molecular chaperones”, and they have additional essential functions in normal cell conditions involving many regulatory pathways.

1.2 Heat Shock Protein Families

Heat shock family members have been identified and named by their molecular weight in kDa such as 70-kDa family of Hsps (Hsp70s) or 40-kDa family of Hsps (Hsp40s). The major families of heat shock proteins are Hsp40, Hsp60, Hsp70, Hsp90, Hsp100 and the small Hsps (sizes below 30 kDa).

Members of the heat shock proteins function as a network to perform variety of cell processes. These proteins play several roles such as assisting membrane translocation, folding of nascent proteins or refolding of misfolded proteins. Many heat shock proteins work together in co-chaperone complexes such as Hsp70-Hsp40 complex (bacterial DnaK/DnaJ) or Hsp60-Hsp10 complex (bacterial GroEL/GroES). Table 1.1 shows major heat shock protein families, their eukaryotic localizations and their prokaryotic homologs.

Table 1.1 : Major heat shock protein families.

Protein family	Prokaryotic family members	Eukaryotic localization
Small heat shock proteins (e.g.Hsp27)		Cytosol /nucleus
Hsp40	DnaJ	Cytosol / nucleus/ ER
Hsp60	GroEL (co-chaperone: GroES)	Mitochondria / chroloplast
Hsp70	DnaK(co-chaperones: DnaJ,GrpE)	Cytosol/ nucleus/ER/ mitochondria/ chroloplast
Hsp90	HtpG	Cytosol
Hsp100	ClpB, ClpA, ClpX	Cytosol

1.3 Heat Shock Protein 70 (Hsp70)

Hsp70 is one of the most ubiquitously expressed family member and is an evolutinarily highly conserved protein among all species from bacteria to human. For instance, Hsp70 proteins of *Drosophilia* have about 70% amino acid identity its yeast and *Escherchia coli* homologs (Craig et al., 1982; Lindquist, 1986). Amino acid similarity between DnaK, *E. coli* homolog of Hsp70, and human Hsp70 is about

50% and especially for some domains this amino acid similarity is around 96% with some domains (Schlesinger et al., 1990).

Hsp70 plays an essential role in the cell homeostasis. They provide "conformational homeostasis" of cellular proteins by getting involved in folding of non-native proteins. Cellular stress conditions such as high temperature, oxidative stress, chemical perturbations etc. induce the synthesis of Hsp70 proteins in order to ensure cell homeostasis. In accordance with this view, dysregulation of Hsp70 is associated with numerous diseases such as immunologic diseases, neurodegenerative diseases, cardiovascular diseases and cancer. For instance, in most of the neurodegenerative diseases such as in Alzheimer's disease, Parkinson's disease, Huntington's disease, Wilson's disease, Alexander's disease and prion-related human syndromes, neuronal cells suffer from the formation of great amount of protein aggregates. Hsp70 is one of the heat shock protein that struggles against protein aggregation (Mayer et al., 1991) and several studies have demonstrated that overexpression of Hsp70 has a potential therapeutic effect in the treatment of neurodegenerative disorders (Hansson et al., 2003; Hay et al., 2004).

1.4 Heat Shock Response and Synthesis of Hsp70

In prokaryotes, DnaK is constitutively transcriped by a single gene. Under stress conditions such as elevated temperature, the rate of expression of DnaK increases within a few minutes. This stress response is regulated by an σ transcription factor, σ^{32}, which is the product of the rpoH (htpR) gene. At optimal growth conditions, DnaK is associated with σ^{32} in order to prevent the formation of RNA polymerase-σ^{32} complex and promotes the degradation of σ^{32} (Liberek et al., 1992). Under stress conditions, σ^{32} dissociates from DnaK and activates the transcription of heat shock genes. On the other hand, absence of the stress conditions and enough synthesis of DnaK cause the degradation of σ^{32} (Straus et al., 1987).

In eukaryotes, more than one gene encodes for Hsp70 proteins and the human Hsp70 family consists of at least eight gene products (Daugaard et al., 2007). Heat shock response in eukaryotes is regulated by heat shock factor 1 (HSF 1). At optimal growth conditions, HSF1 appears as an inert monomer in the cell because Hsp90, Hsp70, and Hdj1 prevent the formation of the HSF1 trimers. Under stress conditions, liberation of HSF1 monomers leads to phosphorylation and formation of the HSF1

trimers. After trimerisation and phosphorylation, HSF1 trimers enter the nucleus and bind to the heat shock elements (HSE) to induce the promoter of heat shock genes (Morimoto, 2002). During the attenuation of stress response, trimers of HSF1 are negatively regulated by the HSF binding protein 1 (HSBP1) which cause dissociation of HSF1 trimers and appearance of HSF1 inert monomers.

1.5 Functions of Hsp70

Hsp70 is a molecular chaperone that function both under normal and stress conditions. Hsp70 plays various roles; they assisting in the folding of newly synthesized proteins, the prevention of aggregation and refolding of misfolded proteins, the degradation of damaged proteins. In addition, Hsp70 provides the translocation of organellar and secretory proteins through membranes and the control of the activity of regulatory proteins (Bukau and Horwich, 1998; Naylor et al., 2001).

In eukaryotes, Hsp70s function in different cell compartments such as cytosol, nucleus, ER and mitochondria, and they perform different functional properties in these compartments. For instance, in ER, misfolded proteins are refolded or degraded by ER's Hsp70, and accumulation of the misfolded proteins enhances the level of Hsp70 (Mori et al. 1996). In mitochondria, mitochondrial Hsp70 (mHsp70) promotes the protein transport across the inner membrane and provides folding of mitochondrial proteins in the matrix (Horst et al. 1997). Hsp70 also plays role in apoptosis and immune response by acting as an anti-apoptotic protein through inhibition of caspase-dependent and caspase-independent pathways of apoptosis (Jäättelä et al. 1998).

1.6 Structural Analysis of Hsp70

All members of the Hsp70 family contain two major domains; a highly conserved 44 kDa N-terminal ATPase domain (nucleotide binding domain, NBD) and a 25 kDa C-terminal substrate binding domain which are connected by a highly conserved hydrophobic linker. Initially, X-ray crystallographic and NMR studies with isolated NBD and SBD revealed the structures of these domains individually: These include X-ray crystallographic studies with isolated NBDs of bovine Hsc70 (Flaherty et al., 1990) and human Hsp70 (in ATP and ADP bound forms) (Sriram et al., 1997), X-ray

studies with isolated SBD of *E. coli* DnaK complexed with heptapeptide substrate (NRLLLTG) (Zhu et al., 1996) and NMR studies with isolated 21 kDa SBD of the *E.coli* DnaK (Wang et al., 1998) and isolated 15 kDa SBD of the mammalian Hsc70 (Morshauer et al., 1999). On the other hand, recently X-ray and NMR studies have revealed the two-domain Hsp70 structure: X-ray structure of a truncated bovine Hsc70 (residues 1-554) without a nucleotide (Jiang et al. 2005) and substrate and a truncated *Geobacillus kaustophilus* DnaK (residues 1–509) with ADP nucleotide (Chang YW et al, 2008), a NMR structure of truncated *Thermus thermophilus* DnaK (residues 1-501) in the ATP and ADP bound forms (Revington et al., 2005), X-ray structure of the ATP-bound yeast full-length Hsp110 (Sse1) (Liu et al., 2007) and full-length *E. coli* DnaK (1–638) complexed with ADP nucleotide and a peptide substrate (Bertelsen et al., 2009) have been reported. Model of the overall structure of *E. coli* DnaK is illustrated in Figure 1.1. All of these studies provided information about structures of NBD and SBD in different ligand forms. On the other hand, recent studies with two-domain Hsp70 provided information about the ligand-stimulated allosteric communication between two domains.

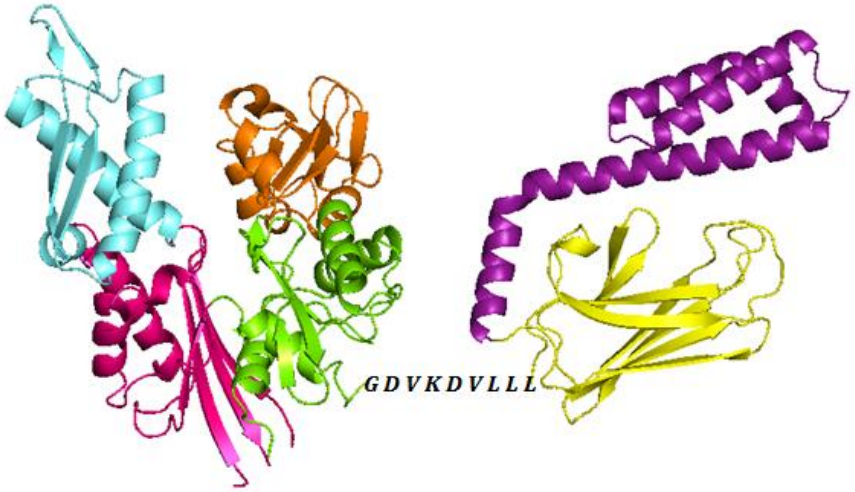

Figure 1.1 : Molecular structure of *E. coli* DnaK. ATPase region [Protein Data Bank ID (PDB ID): 1DKG] and substrate binding region [Protein Data Bank ID (PDB ID): 1DKX] are shown left and right, respectively. Linker region (GDVKDLLL) is shown between two domains.

1.6.1 Structural analysis of the ATPase domain of Hsp70

Crystallographic studies with isolated NBDs demonstrated that ATPase domain of Hsp70 comprises two lobes, I and II which are separated with a deep nucleotide

binding cleft; and each lobe consist of two subdomains, IA-IB and IIA-IIB (Flaherty et al., 1990; Sriram et al., 1997). Crystal structure of bovine Hsc70 complexed with several adenosine nucleotide (ADP, ADP·P_i, ATP) demonstrated that nucleotide is positioned in the active site by the interactions with two β- and γ-phosphate-binding loops and a hydrophobic adenosine binding pocket and connected with IIA and IIB subdomains via hydrogen bonds (Flaherty et al., 1990). Similar structure and binding motifs were also obtained for the ATPase domain of human Hsp70 (Sriram et al., 1997). In addition, Sriram et al. human Hsp70 crystal structure revealed additional two calcium ions in the crystal structure (Sriram et al., 1997). They found that one of these calcium ions is used instead of a magnesium ion for the ATP hydrolysis (this Mg^{2+} ion was shown to be critical for ATP hydrolysis in the crystal structure of bovine Hsc70) (Sriram et al., 1997). On the other hand, the other calcium ion has no known function but it is thought that it may play a role in protein stability (Sriram et al., 1997). In addition, X-ray structures of Hsp70 homologs bound to their respective nucleotide exchange factors (NEF) [DnaK/GrpE, Hsc70/Bag, Hsp70/Hsp110] demonstrated that nucleotide exchange leads to an opening of the nucleotide-binding cleft by a rotation of subdomain IIB about 10 to 30° (Harrison et al., 1997; Sondermann et al., 2001; Polier et al., 2008; Schuermann et al., 2008) (Figure 1.2). It was suggested that a hinge region localized at the interface between the IIA and IIB subdomains controls this rotation (Bhattacharya et al., 2009; Woo et al., 2009).

Crystal studies with NBD did not show any obvious difference in the overall conformation of the NBD between the ATP- and the ADP- bound states (Flaherty et al., 1990; O'Brien et al., 1996). However, recent NMR studies with bovine Hsc70 NBD have demonstrated that ATPase domain has high flexibility and alters its conformation in different nucleotide binding states (Zhang and Zuiderweg, 2004). Movements of the subdomains toward each other cause nucleotide binding cleft to open and close (Zhang and Zuiderweg, 2004). The opening of the nucleotide binding cleft becomes largest in the nucleotide-free state and gradually decreases in the ADP, ADP+P_i and ATP bound states, respectively (Gässler et al. 2001). NMR studies with NBD of Hsc70 have also shown that chemical shift changes occur between the ADP·P_i-bound and ATP-bound states. Furthermore, chemical-shift analyses with NBD of the *E. coli* DnaK also indicated that large chemical shift perturbations occur during the nucleotide exchange particularly in the two α-helices of subdomain IIB.

These two α-helices are located at the interface between the IIA and IIB which is the same location with hinge region (Zhuravleva and Lila, 2011). Thereby, this study suggested that these chemical-shift analyses were consistent with X-ray structures of NEF-bound Hsp70's which showed rotation of IIB subdomain upon nucleotide exchange (Figure 1.2) (Zhuravleva and Lila, 2011).

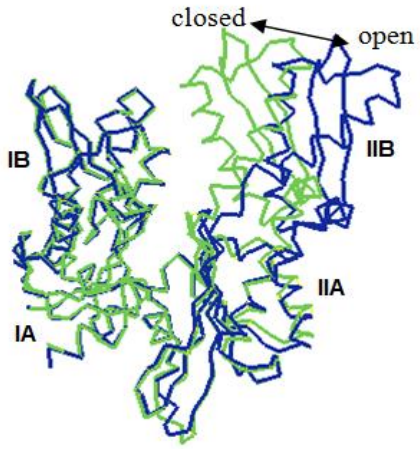

Figure 1.2 : X-ray structures of DnaK homologues. The closed form of *Bos taurus* Hsc70 NBD [Protein Data Bank ID (PDB ID): 1KAX] (green), the open form of the complex of yeast Sse1 with the Bos Taurus Hsc70 NBD [Protein Data Bank ID (PDB ID): 3C7N] (blue).

Recent full-length Hsp70 crystal and NMR structures also provided interesting results for the ATPase domain conformations: One of the obtained X-ray structure of ATP-bound yeast full-length Hsp110 (Sse1) which has strong homology with Hsp70 provides a model for the Hsp70 ATP-bound conformation. According to this crystal structure, two domains of Hsp110 are locked in the ATP-bound state (Liu et al. 2007). In addition, it was shown that hydrophobic cleft between subdomains IA and IIA of Hsp110 is open in the ATP-bound state (Liu et al. 2007). Open cleft was also shown in the NMR studies with *E. coli* DnaK (1–552) and *T. thermophilus* DnaK in ATP-bound form (Swain et al., 2007; Bhattacharya et al., 2009). NMR studies with *T. thermophilus* DnaK have also demonstrated that major rotations occur in the NBD subdomains IA and IIA with respect to each other when changing from the AMPPNP (analog of the ATP) state to the ADP state. On the other hand, NMR studies with *E.coli* DnaK (1-552) in the ADP-peptide state and full-length *E. coli* DnaK (1-638) complexed with ADP nucleotide and a peptide substrate have demonstrated that two

domains do not interact and behave as independently (Swain et al., 2007; Bertelsen et al., 2009). Consequently, these studies suggest that conformational changes occur in the Hsp70 between the ADP and ATP state; in the ADP-bound state two domains behave as independently and linker moves freely but in the ATP-bound state two domains becomes docked and this conformation is formed by rotation of the NBD subdomains which place the linker in the hydrophobic cleft between subdomains IA and IIA and force the SBD to dock on the IA area.

1.6.2 Structural analysis of substate binding domain

Substrate binding domain of Hsp70 was determined with several X-ray and NMR studies (Zhu et al. 1996; Wang et al., 1998; Morshauer et al., 1999). According to these studies, substrate binding domain consists of a β-sandwich subdomain and an α-helical subdomain.

β-sandwich subdomain consists of two layer β-sheets, each sheet contains four antiparallel strands. Substrate binding cavity is formed by β-sheets with inner and outer loops. α-helical subdomain consists of five helices, two of them are helix A and helix B which interact with β-sandwich subdomain, and it is thought that helix B acts as a lid and control the entry of the substrate to the hydrophobic binding cavity. α-helical lid and substate binding cavity are shown in Figure 1.3. Formation of the salt-bridge and hydrogen bonds between helix B and outer loops leads to the closing of the substrate binding cavity. Because of the hydrophobic structure of the binding cavity, Hsp70 prefers a peptide segment which includes hydrophobic residues, especially leucine (Richarme and Kohiyama, 1993). On the other hand, other helices C, D and E form a hydrophobic core with unknown function.

Figure 1.3 : Substrate binding domain of Hsp70 [Protein Data Bank ID (PDB ID): 1DKZ]. This figure shows α-helical lid, substrate-binding cavitiy and extended linker that can dock into the hydrophobic pocket of the ATPase domain.

1.7 Molecular Mechanism of Hsp70 and its Co-chaperones in Functioning Cycle

According to X-ray structure of the ADP-bound DnaK and additional literature studies; there are no interactions between the ATPase domain and the substrate-binding domain (Swain et al., 2007; Chang YW et al, 2008; Bertelsen et al., 2009). At the ADP-bound state of Hsp70, two domains behave independently and they are only connected with a fully extended linker thus this conformation is reffered as the open conformation. On the other hand, the binding of the ATP lead to conformational changes in the Hsp70. At the ATP-bound states of Hsp70, two domains are tightly docked thus this conformation is reffered as the closed conformation (Figure 1.4).

ATP binding stabilizes the ATPase domain and decreases the substrate binding affinity thus, causes the release of the substrate. In the ATPase cycle, substrate binding triggers the hydrolysis of the ATP to ADP and hydrolysis of ATP leads to a conformational rearrengement in both domains. In the substrate-binding domain, hydrolysis of the ATP increases the substrate-binding affinity and leads to the closing of the substrate binding cavity via α-helical lid conformation rearragements. Folding process occurs when substrate is locked in the substrate binding cavity.

In vivo, there are two main co-chaperones which stimulute the ATPase cycle of Hsp70. One of them is Hsp40 (DnaJ) which plays an important role in the regulation of comformational transmission and increases the rate of the ATP hydrolysis. This

co-chaperone is required for Hsp70 function because intrinsic ATP hydrolysis rate is very low and inadequate to perform the function of Hsp70 *in vivo*. For instance, in *E. coli*, DnaJ has an essential role in the bacteriophage λ DNA replication and DnaK requires DnaJ to function in the activation of helicase (DnaB) which initiates bacteriophage λ DNA replication (Alfano and McMacken, 1989).

According to NMR and X-ray structures, DnaJ consists of a J-domain, a glycine-phenylalanine rich region, a cysteine rich segment and a C-terminal region. J-domain is the most important part of the DnaJ for the stimulation of ATP hydrolysis. J-domain of DnaJ binds to the ATPase domain. On the other hand, C-terminal region of DnaJ interacts with the substrate and enables the substrate to bind to the substrate binding cavity of DnaK.

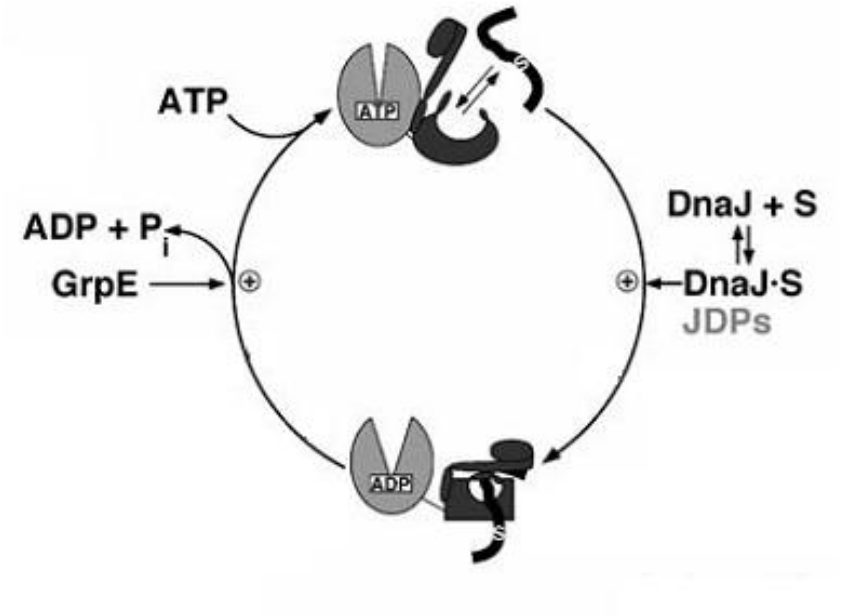

Figure 1.4 : Functional cycle of the Hsp70: ATPase domain of Hsp70 is drawn in grey and the substrate in black. This figure is adapted from Bukau et al. 2004.

The other main co-chaperone of the Hsp70 is the GrpE, which acts as a nucleotide exchange factor (NEF). X-ray structures of Hsp70 which complex with NEF have demonstrated that NEF enhances the dissociation rate of ADP from Hsp70. This co-chaperone binds to the ATPase domain to assist the dissociation of ADP from Hsp70 and thereby, it leads to the release of substrate (Harrison et al., 1997).

1.8 Interdomain Linker of Hsp70 and Allosteric Communication

The ATPase domain and the substrate-binding domain of Hsp70 are connected by a hydrophobic and highly conserved flexible linker, which acts as a molecular switch and plays a key role in the allosteric communication. This interdomain linker can adopt different conformational forms in the functioning cycle of Hsp70 depending on the ligand state. In the ADP-bound state, fully extended linker leads to separation of the domains, on the other hand in the ATP-bound state, linker contact with the hydrophobic cleft between subdomains IA and IIA of ATPase domain and leads to docking of the domains.

Mutational studies with the linker region demonstrated important role of the linker in the allosteric communication between two domains. In one study, four hydrophobic residues of the DnaK linker ^{389}(VLLL)^{392} were replaced by alanine ^{389}(AAAA)^{392} and two leucine residues ^{390}(LL)^{391} were replaced by two aspartate ^{390}(DD)^{391}, respectively (Laufen et al., 1999). These mutations abolish DnaJ stimulation and dramatically decreased the substarate-stimulated ATPase activity which would explain the essential role of the linker in the interdomain communication (Laufen et al., 1999). In another study, one of the linker residues Asp393 (D393) was replaced by an arginine and an alanine. These replacements led to reduction in the ATP-stimulated substrate dissociation rate and also reduction in the DnaJ and substrate stimulated ATPase activity (Vogel et al., 2006a). These mutations also reduced the ATPase activity in the DnaK (2-393) construct (Vogel et al., 2006a). This result is also a strong evidence for linker having an essential role in the allosteric communication (Vogel et al., 2006a). Recently, a similar mutational study has been done to better understand the role of the linker in the allosteric coupling mechanism. In this study, Val389 and Leu391 redidues which are also located in the linker were replaced by an aspartate and an alanine. These mutations caused a loss of *in vivo* function of DnaK. *In vitro* analyses also showed that these mutations led to reduction of the refolding activity and peptide-stimulated ATPase activity (Kumar et al., 2011).

Studies with isolated DnaK ATPase domain with and without the linker showed that linker alone can stimulate the ATPase activity of the ATPase domain and mimic the substrate-stimulated ATPase activity of full-length DnaK (Swain et al., 2007). All of these studies emphasize the importance of the linker in the interdomain

communication. On the other hand, several crystallographic and NMR studies with Hsp70 homologs have demonstrated the conformational differences between different ligand forms of Hsp70s (Jiang et al. 2005; Liu et al. 2007; Swain et al., 2007; Chang YW et al, 2008; Bertelsen et al., 2009; Bhattacharya et al., 2009). Based on these studies, it is proposed that linker plays an essential role in these conformational differences. But, due to the absence of X-ray structure of the full-length Hsp70 comprising different ligand forms (ATP, ADP or nucleotide free and peptide substrate), molecular mechanism of the interdomain communication can not be understood completely.

1.9 Mutational studies done so far on the ATPase domains of Hsp70

Initial crystal structural analyses for NBD revealed several acidic residues that are important for catalysis in the active region of the ATPase domain. For instance, mutational analyses of some of these acidic residues in Hsc70 (1–386) NBD (Asp10; D10S-D10N, Asp199; D199S-D199N and Asp206; D206S-D206N) have led to 10–100-fold lower ATPase turnover rate (Wilbanks et al., 1994). Crystallographic study for Hsc70 NBD (1–386) demonstrated that conserved Glu175 (E175) residue (E171 for DnaK) connect the hinge region that is proposed to be responsible for the movement of subdomains to in the Mg^{2+}/ATP-bound form (Holmes et al., 1993). Mutational analysis with this glutamate residue has demonstrated that this residue is catalytically essential. Single point mutations such as E171A, L, K for the full-length DnaK and E175Q, S for Hsc70 (1–386) reduced the affinity of DnaK and Hsc70 for ATP (Wilbanks et al., 1994; Buchberger et al., 1994). Mutations in this glutamate residue also caused a failure in the substrate-stimulated ATPase activity, decreased refolding activity, thus formating a non-functional DnaK (Buchberger et al., 1994).

Lys71 (K71) is another residue which is also located in the active region. It was proposed that, K71 is a catalytic residue by positioning a water molecule for the nucleophilic inline attack on the γ-phosphate (O'Brien et al., 1996). Mutagenesis of K71 of Hsc70 (1–386) to glutamic acid, alanine, and methionine led to significant reduction in the ATPase activity. As a result, this mutational analysis demonstrated that this residue is essential for the chemical hydrolysis of ATP (O'Brien et al., 1996).

Crystal structure of Hsc70 NBD also showed that Thr204 (T204) is located in the active site and hydroxyl of T204 is located close to the γ-phosphate of ATP (Flaherty et al., 1990). Therefore, this residue was proposed to participate in the ATP hydrolysis. However, mutational analysis at this residue (threonine to valine, T204V and threonine to glutamic acid, T204E) demonstrated that this residue affects structure of the active site but it is not essential for ATP hydrolysis (O'Brien and McKay, 1993).

According to X-ray structure of Hsc70 NBD that Pro147 (P147) (P143 for DnaK) is located in the ATP binding site and connected to key residues such as K71 and E171 (Flaherty et al., 1990). Mutational analyses with this residue demonstrated that replacements of P143 with glutamic acid and alanine also reduced the basal ATP hydrolysis rate of DnaK. Additionally, mutations in this residue also reduced DnaJ and substrate situmulated ATPase rate. These results were explained with a hypothesis that P143 do not play a direct role in catalysis but it is important for the positioning of the catalytic residue K70 (K71 in Hsc70) (Vogel et al., 2006a). In addition, studies showed that mutagenesis of R151 (R151) to alanine and lysine increased the basal ATP hydrolysis rate. But these R151A and R151K mutants led to reduce the peptide dissociation rate and they could not be stimulated neither with DnaJ nor with substrate. In the light of these results, a hypotethical model is proposed for the allosteric regulation of Hsp70. P143 residue acts as a switch between E171, K71 and R151 residues and R151 provides allosteric communication between two domains by the help of P143 (Vogel et al., 2006a).

Lys155 (K155), Arg167 (R167), Asp388 (D388) and Asp393 (D393) residues were used in a study to propose a model for allosteric regulation (Vogel et al. 2006b). Positively charged K155 and R167 are located at the surface of subdomain IA in the ATPase domain of DnaK. Replacement of the K155 by alanine (K155A) and aspartic acid (K155D), R167 by alanine (R167A) and aspartic acid (R167D) led to a reduction in the rate of the ATP-stimulated substrate dissociation (Vogel et al. 2006b). These mutations also reduced intrinsic ATPase activity and DnaJ and substrate stimulated activity. In contrast, these mutations in the DnaK (2-393) construct, which contains linker region, led to increased ATPase rate. These results demonstrated that these positively charged residues are essential for the allosteric communication but negatively charged ones modulate the ATPase activity.

According to crystal structure, these residues are located near Pro143 and it was proposed that these residues trigger the proline switch to conformational transition for ATP hydrolysis which would explain the role of these residues in the allosteric communication. In addition, mutations in the negatively charged D393 residue, which is located in the linker region, led to a reduction in the rate of the ATP-stimulated substrate dissociation and intrinsic ATPase activity and also DnaJ and substrate stimulated activity. Replacement of D193 by arginine and alanine also led to reduction of the ATPase rate in the DnaK (2-393) constract. These results suggested that linker is responsible for triggering the ATP hydrolysis and this is a strong evidence for the linker playing an essential role in the interdomain communication. On the other hand, replacement of the D388 by arginine did not lead to any significant change in the ATPase and peptide dissociation rate suggesting that this residue is not essential for the allosteric communication (Vogel et al. 2006b).

Another mutational study in DnaK revealed the possible relationship between ATPase rate and chaperone activities (Chang et al., 2010). This study showed that mutations in the Glu171 (mutant form: E171S) and Thr199 (mutant form: T199A) residues which are located in the ATP binding cleft led to defects in the DnaJ, GrpE and substrate stimulated ATPase activities. These mutants are also unable to refold the substrate. In contrast to these mutations, this study also demonstrated that mutations in some residues did not show a strong correlation between ATPase rate and refolding activity (Chang et al., 2010). For instance, T12A had increased ATPase rate but this increased rate did not lead to a higher refolding activity. On the other hand, R56A had normal ATPase rate with wt DnaK, but decreased refolding activity. In addition, some mutants such as F67L, P90A, F91A, E230Q, D231N and K263A showed low ATPase rates, but normal refolding activity. F67L, P90A, F91A mutants also led to increased flexibility in DnaK and these residues are located near to the K70. According to these results, it was proposed increased flexibility disrupt the positioning of K70 which would explain the low ATPase rate. In addition, E230 and D231 residues are located in the hinge region which is thought to be responsible for rotation of IIB subdomain upon nucleotide exchange. Thereby, E230Q and D231N mutants were not stimulated by GrpE (NEF) as expected. In conclusion, weak correlation between ATPase rate and refolding activity demonstrated that enzymatic activity of Hsp70 is not directly linked to substrate folding (Chang et al., 2010).

1.10 Aim of the study

Hsp70 is a ubiquitous molecular chaperone, which plays essential roles in the cell homeostasis by getting involved in several cell processes. Disregulation of Hsp70 is associated with many diseases such as immunologic diseases, neurodegenerative diseases, cardiovascular diseases and cancer. However, allosteric mechanism of the Hsp70 at the molecular level is not completely understood. Once it is delineated, new therapeutic approaches can be assessed in Hsp70 involved disease treatment.

Previous studies showed that the linker region between two domains plays an important role in the allosteric communication (Laufen et al., 1999; Vogel et al. 2006; Swain et al., 2007; Kumar et al., 2011). When linker interacts with NBD, studied by the construct containing the entire linker, DnaK(1-392), an enhanced ATPase rate is observed compared to the construct lacking the conserved linker region, DnaK(1-388) (Swain et al. 2007). This enhanced ATPase rate explained by an adoptation of NBD to a closed conformation. In addition studies of Dinler et al. (2007) have also demonstrated pH-dependent ATPase activity in the peptide-bound full-length DnaK and DnaK (1-392) construct. In this study, we aimed to understand dynamics and allosteric mechanim of ATPase domain by pinpointing critical residues that are present in DnaK(1-388) construct. According to our hypothesis, it was thought that when linker region binds to the hydrophobic cleft between subdomains IA and IIA of ATPase domain, a network is formed among His226, Asp85 Thr225 and Arg71 residues (Figure 1.5) which may have critical roles in the ATPase allostery. In this study, to understand the ATPase domain dymanics in the unstimulated state linkerless version of ATPase domain, DnaK(1-388), was used. Based on the hypothesis, we did mutagenesis on His226, Thr225, Asp85 and Arg71 sites and investigated their role in the activity and stability of the ATPase domain. To declare the effects of both charge and size differences on the ATPase activity, His226 was replaced by alanine and phenilalanine; Asp85 was replaced by alanine and glutamic acid, Thr225 and Arg71 were also replaced by alanine. Based on previous studies which have demonsrated pH dependent ATPase activity, all ATPase assays were performed between pH 5.5 and 8.5 to investigate the effects of these residues in the pH-dependent ATPase activity. In addition, mutational studies were also performed for His295 and it was mutated to an aspartic acid which will cause an opposite effect due to the charge inversion. This residue is seleceted to investigate its

possible effect on the pH-dependent ATPase activity. In conclusion, we proposed that mutational studies with these residues will provide deep insight into dynamics of the ATPase domain and allosteric mechanism of Hsp70.

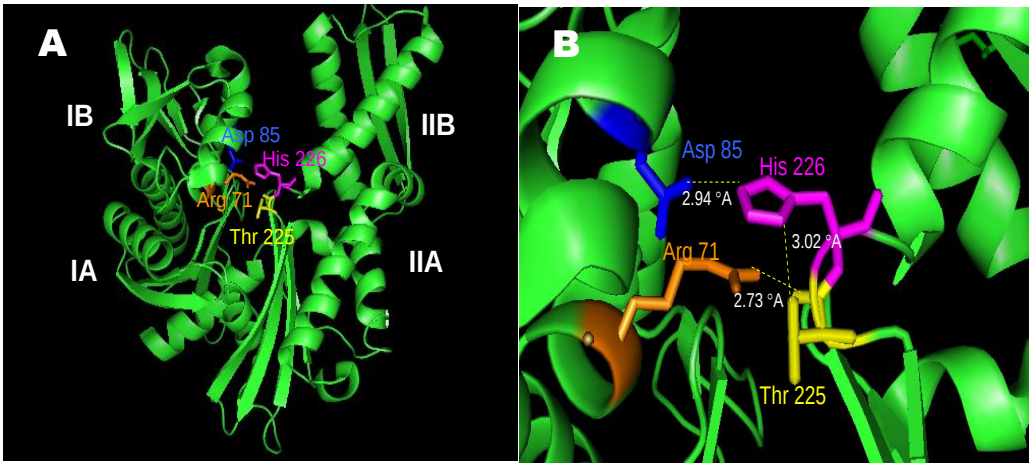

Figure 1.5 : Hypothetical network is shown in the crystal structure of ATPase domain. (A) Locations of Asp85, His226, Arg71 and Thr255. (B) Distances between amino acids in the hypothetical network.

2. MATERIALS & METHODS

2.1 Materials

2.1.1 Laboratory equipments

Equipments used in the study are given in the Appendix A.

2.1.2 Chemicals and enzymes

Chemicals and enzymes used in the study are given in the Appendix B.

2.1.3 Commercial kits

Commercial kits used in this study are given in the Table 2.1.

Table 2.1 : Commercial kits used in the study.

Kit	**Supplier Company**
QuikChange Site-Directed Mutagenesis Kit	Stratagene 200519
QIAquick PCR purification kit	Qiagen, 28104
QiaPrep Spin Miniprep Kit	Qiagen, 27106

2.1.4 Bacterial strains

In this study, *Escherichia coli* XL-1 Blue strain [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* F′ *proAB lacI*q*Z*Δ*M15* Tn*10* (Tetr)] and *Escherichia coli* BB1553 strain [MC4100 _dnaK52::cm^R sidB1] (Stratagene) bacterial strains were used.

2.1.5 Buffer and solutions

Preparation of buffer and solutions are explained in Appendix C.

2.2 Methods

2.2.1 Site-directed mutagenesis

2.2.1.1 Mutant strand synthesis reaction

Point mutations in the DnaK ATPase domain were obtained by Quick Change Site-Directed Mutagenesis Kit (Stratagene). First step of the site-directed mutagenesis method is PCR which was carried out using *PfuTurbo* DNA polymerase. *PfuTurbo* DNA polymerase is a highly thermostable DNA polymerase that provides high-fidelity PCR amplification by its 3'=>5' exonuclease activity (proofreading). In the amplification reaction, plasmid pMS-DnaK(1–388) was used as a template which was created from pMS-DnaK plasmid by PCR (Montgomery et al., 1999). Mutagenesis PCR was performed in 50 µl reaction mixture containing 50 ng dsDNA template, 2.5 U/µl *PfuTurbo* DNA polymerase, 10X *PfuTurbo* DNA polymerase reaction buffer, dNTP mix and 125 ng primers. Mutagenesis primers and PCR cycling parameters used in the study are given in Table 2.2 and Table 2.3, respectively.

Table 2.2 : Mutagenesis primers used in the study.

Primer Name	Primer sequence (5'→ 3')	Tm	% G/C
H226A_F	GGCAACCAACGGTGATACC**GC**CCTGGGGGGTGAAGA	67	63
H226A_R	TCTTCACCCCCCAGGGCGGTATCACCGTTGGTTGCC	67	63
H226F_F	GGCAACCAACGGTGATACC**TT**CCTGGGGGGTGAAGA	65	58
H226F_R	TCTTCACCCCCCAGGAAGGTATCACCGTTGGTTGCC	65	58
T225A_F	GCAACCAACGGTGAT**G**CCCACCTGGGGG	64	68
T225A_R	CCCCCAGGTGGGCATCACCGTTGGTTGC	64	68
D85A_F	CGAAGAAGTACAGCGTG**CT**GTTTCCATCATGCCGT	62	51
D85A_R	ACGGCATGATGGAAACAGCACGCTGTACTTCTTCG	62	51
D85E_F	GAAGAAGTACAGCGTGA**G**GTTTCCATCATGCCGTT	61	49
D85E_R	AACGGCATGATGGAAACCTCACGCTGTACTTCTTC	61	49
H295D_F	GCGACCGGTCCGAAA**G**ACATGAACATCAAAGTG	65	52
H295D_R	CACTTTGATGTTCATGTCTTTCGGACCGGTCGC	65	52
R71A_F	CGCAAAACACTCTGTTTGCGATTAAA**GC**CCTGATTGGTCGCC	64	50
R71A_R	GGCGACCAATCAGGGCTTTAATCGCAAACAGAGTGTTTTGCG	64	50

Table 2.3 : PCR cycling parameters for the mutagenesis method used in this study.

Segment	Cycles	Temperature	Time
1	1	95°C	30 sec
2	18	95°C	30 sec
		60°C	1 min
		68°C	7 min

2.2.1.2 Agarose gel electrophoresis for the detection of PCR amplification

PCR amplification was checked by electrophoresis of 10 µl of the product on a 1% agarose gel. To prepare 1% agarose gel, 0.35 g agarose was dissolved in 40 ml 1X TBE buffer. For each sample, 10 µl of PCR amplicon was mixed by 1 µl of 6X loading dye and 1 µl of SYBR green stain which provide visualization of DNA within agarose gels under UV light. GeneRuler 1 kb Plus DNA Ladder (Fermentas) was used to estimate the PCR amplicon size. Electrophoresis was performed for 35 minute at 120V. Agarose gels were visualized by UV light transilluminator and photographed by a connected camera with UV PhotoMW software.

2.2.1.3 Digestion of template DNA

Following the PCR amplification step, a digestion step was performed using *Dpn I* restriction enzyme. The *Dpn I* endonuclease (target sequence: 5′-Gm6ATC-3′) specifically digests the methylated and hemimethylated DNA. Template plasmid DNA had been isolated from a dam methylated *E. coli* strain (dam^+) and sensitive to *Dpn I* digestion. Therefore, *Dpn I* was used in order digest parental supercoiled dsDNA template to select for new synthesized mutated DNA. At this step, 1 µl *Dpn* I restriction enzyme (10 U/µl) were added to each reaction sample and each reaction was incubated at 37°C for 1 hour.

2.2.1.4 Purification of the PCR product

After PCR and digestion steps, an additional purification step was applied to remove all enzymatic reactions from DNA. PCR products were purified using QIAquick PCR Purification Kit (Qiagen). In order to purification, 5 volumes of binding buffer (3 M guanidine-thiocyanate, 10 mM Tris-HCl, 5% ethanol (v/v), 2 mg RNase) were

added to 1 volume of the each PCR sample. After mixing sample well, PCR sample was transferred into a QIAquick spin column and centrifuged for 1 minute at maximum speed. Then flow-through was discarded and 750 µl wash buffer (20 mM NaCl, 2 mM Tris-HCl, 80% ethanol) was added. Sample was centrifuged for 1 minute at a maximum speed. After discarding the flow-through, an additional centrifugation was applied for 1 minute at maximum speed to remove the residual wash buffer. Then, QIAquick spin column was placed in a clean 1.5 ml micro centrifuge tube. Finally, 50 µl elution buffer (10 mM Tris-HCl, pH 8.5) was added to the column and sample was centrifuged for 1 minute at maximum speed.

2.2.1.5 Transformation of mutated DNA into XL1-Blue supercompetent cells

Incorporation of mutagenic primers generates mutated plasmids which contain nicked circular strands. Therefore, new synthesized plasmids were transformed into XL1-Blue supercompetent cells to repair nicked strands. Nicked DNA was introduced into *E. coli* XL1-Blue supercompetent cells using chemical transformation method which was applied with heat shock. Transformation was performed as following protocol: The competent cells were taken from at -80^{o}C and thawed. 2 µl of mutated plasmid DNA was added to 20 µl of competent cells and swirled gently. Then, the eppendorf tube containing the cells and DNA was incubated on ice for 30 minute. Heat shock process was applied by putting the cells in heat block at 42^{o}C for 45 second and putting on ice for 2 minute. Then, 0.25 ml of LB medium was added to competent cells and eppendorf tube was incubated at 37^{o}C for 1 hour with shaking at 225-250 rpm. Finally, the cells were spread on LB agar plate containing the appropriate antibiotic (ampicilin) and the plate was incubated at 37^{o}C overnight for 16 hour.

2.2.1.6 Plasmid DNA preparation

Single colony of *E. coli* XL1-Blue cells from overnight grown plate was taken into 5 ml LB medium containing the appropriate antibiotic (ampicillin). Then, it was overnight at 37^{o}C with shaking at 225-250 rpm. The next day after incubation, small scale plasmid DNA isolation was performed using QIAPrep Spin Miniprep Kit (Qiagen) according to the following protocol: 5 ml culture was centrifuged for 10 minute at 5100 rpm. Then, the supernatants were discarded. Bacterial cell pellet was

resuspended in 250 µl Buffer P1 which contains RNAse A and transferred into a micro centrifuge tube. Then, to lyse the cells, 250 µl P2 buffer was added and the tube was inverted gently 4-6 times. 350 µl N3 buffer was put into the mixture and immediately the tube was again inverted 4-6 times. The mixture tube was centrifuged for 10 minute at a maximum speed. The supernatant was transferred to the QIAprep column by pipetting. Then, centrifugation was applied again for 1 minute and supernatant was discarded. The QIAprep column was washed by adding 0.75 ml PE buffer and centrifuged for 1 minute. Flow-through was discarded and an additional centrifugation step was applied for 1 minute. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. Finally, to elute DNA, 50 ML EB buffer (10mM Tris-Cl pH-8.5) was added to the center of the Qiaprep column, and the DNA solution was obtained after centrifugation for 1 minute at a maximum speed. Isolated plasmid DNA was stored at -20^{o}C for further use.

2.2.1.7 DNA sequencing

Following the mutagenesis step, selected clones were sequenced to verify the presence of the desired mutations. Selected clones contain the desired mutations. DNA sequencing was performed by AKA Biotechnology (Istanbul, Turkey). Sequencing primers used in the study are shown in Table 2.4.

Table 2.4 : Sequencing primers used in the study.

Primer Name	Primer sequence (5'→ 3')	Tm
Forward Primer	CCGTACTATCGCGGTTTATGACCT	52.3
Reverse Primer	AGCGGAAGACAGTTCGATTTTCG	50.2

2.2.1.8 Analysis of the sequencing results of mutant *dnaK(1-388)* genes

Analysis of sequence results was performed using EMBOSS Needle-Pairwise Sequence Alignment Tool (available online at this web address: http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html).

2.2.2 Expression and purification of DnaK proteins

2.2.2.1 Preparation of competent *E.coli* BB1553 cells

In this study, *E. coli* BB1553 *dnaK* null cells were used which are unable to grow at high temperatures. These cells were grown at 30^{o}C. This temperature was determined as optimum growth temperature in Bukau and Walker study (Bukau and Walker, 1990). Competent cells that are transiently permeable cells to foreign DNA/plasmid were prepared by chemical method with $CaCl_2$. Chemical competent cell preparation for *E. coli* BB1553 cells was performed as following: BB1553 stock cells were streaked onto LB agar plate and inoculated plate was incubated overnight at 30^{o}C. The next day, one colony was picked from the incubated LB plate and put into a 100 ml LB medium. Then, it was incubated at 30^{o}C for 6-8 hours with shaking at 150 rpm to reach OD_{600} = 0.4 – 0.6. Culture was separated into two pre-chilled sterile 50 ml falcon tubes and placed on ice for 10 minute. Cell cultures were centrifuged for 10 min at 4000 rpm at 4^{o}C and the supernatant was discarded. Then, cell pellets were resuspended in 30 ml ice-cold solution A including 0.02 M $CaCl_2$, 0.08 M $MgCl_2$ and then centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant was again discarded. Pellets were resuspended in 2 ml ice-cold solution B including 0.1 M $CaCl_2$ and 15% glyserol. Finally, 100 µl of resuspension was aliquoted into micro-centrifuge tubes and these aliquots were stored at -80^{o}C.

2.2.2.2 Transformation into the BB1553 competent cells

Both mutant and wild-type pMS-DnaK(1-388) vectors were transformed into BB1553 cells by a similar transformation protocol which is described in the 2.2.1.5 part. Unlike this protocol, BB1553 cells were incubated at 30^{o}C and spreaded on LB agar plate containing ampicillin and chloramphenicol antibiotics.

2.2.2.3 Growth of the BB1553 cells

Both wild-type and mutant plasmids were overexpressed in *E. coli* BB1553 cell strain at 30^{o}C. Growth steps were appllied as the following: After the transformation step one colony was put into a 250 ml LB medium containing appropriate antibiotics (ampicillin and chloramphenicol). Then, this culture was incubated overnight at 30^{o}C. Next day, 50 ml of grown overnight culture was added to 1 L of LB-amp-chl media. Then, culture was incubated at 30^{o}C until cells reached to OD_{600}=1. When

culture reached to OD_{600}=1, 1 ml of culture was taken out to an eppendorf and continued growing without adding IPTG for an uninduced control. Then, 0.2 mM isopropyl-1-thio-β-d-galactopyranoside (IPTG) was added to the 1 L culture for induction. Induced culture was continued to grow for 4 hours. After 4 hours growth, 1 ml of culture was taken out for an induced control. Then, induced and uninduced control cells were spinned down and resuspended in 20-100 µl of either resuspension buffer. Both induced and uninduced controls were later checked by SDS-PAGE. On the other hand, 1 L culture was centrifuged at 5000 rpm for 30 minute. Finally, pellets of the 1 L culture were resuspended in resuspension buffer as the ratio 3 ml/g pellet and the cells were stored at −80°C.

2.2.2.4 Control of induction

Both induced and uninduced resuspended control samples (20-100 µl) were mixed with lysozyme and PMSF. Then, sample mixes were incubated on ice for 30 minute to lyze the cells. 10 µl of samples were mixed with 2X Laemmli sample buffer for SDS-PAGE analysis which was explained below.

2.2.2.5 SDS-polyacrylamide gel electrophoresis of proteins (SDS-PAGE)

SDS (sodium dodecyl sulfate) is an anionic detergent which covers the proteins with negative charges and denatures secondary and non–disulfide–linked tertiary structures. Therefore, SDS-PAGE is a technique that provides to separation of proteins according to their molecular weight. Polyacrylamide gel matrix is formed by free radical oligomerization of acrylamide and bis-acrylamide which forms cross-links between two polyacrylamide molecules. Pore size of the gel is determined by concentrations of acrylamide and bis-acrylamide and varied according to molecular weight of proteins. SDS-PAGE has a discontinuous gel system. Two gel layers are used: running (resolving) and stacking gels. Stacking gel which contains lower acrylamide concentration is cast over the top of the running gel and allows the protein samples to be concentrated into a tight band during before entering the running gel part. In order to separation of 42 kDa DnaK(1-388) proteins, SDS-polyacrylamide gel with 12 % acrylamide concentration was prepared. Initially, 5 ml of %12 running gel was prepared. When the running gel solidified, 2 ml of 5% stacking gel was prepared and poured on top of the resolving gel. Finally, the comb was placed before the stacking part solidified. 10 µl samples containing proteins

were mixed with 2X Laemmli sample buffer. The samples were denaturated for 5 minute at 95ºC. Then, 10 µl of mix was loaded on the wells. As size standard, Unstained Protein Molecular Weight Marker (Thermo Scientific), containing 7 unstained protein bands in the range of 14.4-116 kDa, was loaded on the gel. Electrophoresis was carried out in SDS buffer at 95 V, 200 mA, for 100 minute. After electrophoresis, gel was stained with Coomassie brilliant blue solution for visualization of the bands. Then, gel was destained using the destain solution.

2.2.2.6 Preparation of Cell Extract

Soluble cell extract was prepared by a lysozyme treatment and sonication. This preparation steps were applied as the following; ~ 35 ml aliquot of frozen cell suspension (pellet from 2 L of bacterial culture) were taken from -80^{o}C and thawed. Then, cells were treated with lysosyme (0.2 mg/ml), DNase I (0.15 mg/ml) and several protease inhibitors [PMSF (0.5 mM), leupeptin (5 µg/mL), aprotinin (2.5 µg/mL) and pepstatin A (1 µg/mL)]. This mixture was incubated on ice for 30 minute. Then, sonication was applied roughly 10 times (20 second bursts) on ice. Finally, lysate was centrifuged for 30 minute, at 12.000 rpm and 4^{o}C, and the supernatent was collected for protein purification.

2.2.2.7 Purification of DnaK proteins

Protein purification step was performed with fast protein liquid chromatography (FPLC). FPLC is a technique to separate macromolecules according to their sizes, charge distribution, hydrophobicity, affinities and their solubilities. The FPLC comprises one or two high-precision pumps, a control unit, a column, a detection system (UV or UV/Vis spectrophotometer) and a fraction collector. In this study, in order to purify the wild-type and mutant DnaK(1-388) proteins, two columns were used with FPLC: (1) Diethylaminoethyl (DEAE)–Sephacel column and (2) ATP-agarose column.

First step of DnaK Purification by DEAE–Sephacel column

DEAE Sephacel is an anion exchanger based on beaded form of cellulose. In this column process, negatively charged molecules are attracted to positively charged (DEAE) beads. In the first step of DnaK purification, DEAE–Sephacel column was used to separate DnaK proteins from positively charged proteins. Before starting the

purification, column was cleaned with 4 column volumes of 2 M NaCl solution, 2 column volumes of 50% EtOH, and 2 column volumes of dH_2O, respectively. This cleaning process was applied to remove hydrophobically bound proteins, lipoproteins and lipids and also to prevent cross-contamination. Column was equilibrated with 5 column volumes of Buffer A. After the equilibration step, soluble cell extract was loaded on DEAE Sephacel column. Then, the column was washed with 4 column volumes of Buffer A (20 mM Tris-HCl, 1 mM EDTA) to remove nonspecifically bound proteins. Finally, DnaK proteins were eluted with 100 ml of Buffer B.

Following the DEAE–Sephacel column purification, 100 ml of Buffer B (20 mM Tris-HCl, 1 mM EDTA and 1 M NaCl) fraction containing DnaK proteins was concentrated in an Amicon ultrafiltration cell (Millipore, Billerica, MA). Then, 10 ml of concentrated protein sample was dialyzed with 4 L HMK buffer.

Second step of DnaK Purification by ATP-agarose column

ATP-agarose column (Sigma catalog number A9264) is an affinity column which contains ATP-agarose beads. DnaK proteins bind to these ATP-agarose beads through their ATP binding properties. This second step of DnaK purification was applied as the following protocol: Before starting to the purification, ATP-agarose column was cleaned with 10 column volumes of buffer containing 10 mm EDTA, 100 mm KCl to prevent cross-contamination and equilibrated with 6 column volumes of HMK buffer (20 mm HEPES, 5 mm $MgCl_2$ and 100 mm KCl). Then, dialyzed protein sample was loaded onto the ATP-agarose column. The column was washed with ~3 column volumes of HMK plus 2 M KCl buffer to remove nonspecifically bound proteins. Then, column was washed with 3 column volumes of HMK to re-equilibration. Finally, DnaK proteins were eluted with HEK buffer (20 mm HEPES, 10 mm EDTA and 100 mm KCl)**.**

After the purification, pooled DnaK fraction was concentrated in an Amicon ultrafiltration cell (Millipore, Billerica, MA). Then, purity of the concentrated DnaK fraction was checked by SDS-PAGE. After this step, concentrated DnaK fraction was dialyzed against 4 L HMK buffer. Then, 50 µl of dialyzed protein sample was aliquoted into micro centrifuge tubes and these aliquots were stored at -20^{o}C.

2.2.3 Enzyme coupled ATPase assay

ATPase activity of DnaK proteins was measured using an NADH-coupled ATPase assay. Hydrolysis of ATP to ADP results in conversion of phosphoenolpyruvate (PEP) to pyruvate by pyruvate kinase (PK). The resulting pyruvate is converted to lactate by lactate dehydrogenase (LDH). These coupled enzymatic reactions result in oxidation of NADH to NAD^+. The decrease of NADH at 340 nm enables monitoring the rate of ATP hydrolysis. In this study, ATPase assay was performed by a Biorad Benchmark Plus microplate spectrophotometer and the decrease of NADH in A_{340} was measured for 30 minutes at 30°C. The assays were carried out in a 200 µl sample reaction including following reagents; 5X ATPase buffer [40 mM Hepes, 50 mM KCl, 11 mM $Mg(OAc)_2$], 0.28 mM ATP, 1.02 mM PEP, 5mM DTT, 0.2 mM β-NADH, PK/LDH cocktail (9.94 U/ml PK-14.2 U/ml LDH), 1 µM protein. In addition, an autohydrolysis reaction was also carried out lacking protein samples. Protein concentrations for DnaK(1-388) and mutant proteins were determined with Thermo Scientific NanoDrop 1000 spectrophotometer using an extinction coefficient of 18607 $M^{-1}cm^{-1}$ at 280 nm. Before measurement, reaction samples were incubated for 5 minutes at 30°C. For pH-dependent ATPase assays, pH was always adjusted for each sample using 5X ATPase buffer at different pHs. To calculate the ATPase rate, initially absorbance data was plotted and slope was calculated using a linear regression fit. Then, slope of the autohydrolysis reaction was subtracted from slope of the reaction. As a result, ATPase rate was determined as the following equation (1).

$$\text{ATPase rate } [min^{-1}] = -\frac{dA_{340}}{dt}\,[OD/min] \times K_{path}^{-1} \times moles^{-1}\,ATPase \qquad (1)$$

K_{path} is the molar absorption coefficient for NADH for a given optical pathlength. For 200 µl well sample volume K_{path} is equal to 3,247.

2.2.4 Native gel electrophoresis

Native or non-denaturing gel electrophoresis provides to detection of proteins under native conditions. Different from the SDS-PAGE method, native PAGE does not use any denaturing agent and thus, mobility of the proteins in the native gel does not only depend on their molecular mass but also depends on their shape and native charge. In

this study, DnaK(1-388) wild-type and its mutants were detected by native gel to understand the structural effect of mutations in the presence and absence of ATP. Before running of the native gel, all proteins were incubated at 30^{o}C for 30 minutes. Generally, all proteins were loaded into native gel in the range of concentration from 1μM to 30 μM. 0.28 mM ATP was used for 1μM protein as did in the ATPase assay. ATP concentration was increased as the same proportion with protein concentration. Native gel was prepared as the following; to prepare the 10% running gel, 2 ml of 40% acrylamide-bis solution, 2 ml of 1.5 M Tris-HCl (pH 8.8), 40 μl of APS, 4 μl of TEMED were mixed and dH_2O was added to make a final volume of 8 ml. Then, to prepare the stacking gel, 187.5 μl of 40% acrylamide-bis solution, 500 μl of 0.5 M Tris-HCl (pH 6.8), 16.6 μl of APS, 2.5 μl of TEMED were mixed and dH_2O was added to make a final volume of 2 ml.

2.2.5 Circular dichroism measurements

Circular Dichroism (CD) spectroscopy measures the differential absorption of left and right circularly polarized radiation. CD provides the analysis of secondary structural features of a protein (α-helices and β-sheets). In this study, CD spectroscopy was used to get information about secondary structures of mutant proteins and investigate the possible structural differences between wild-type and mutant proteins. CD measurements were performed using Jasco J-810 spectropolarimeter (Jasco, Easton, MD). CD scans were performed in the "far-UV" spectral region (190-250 nm) using a 0.1 cm cuvette. In addition, thermal melting curve analysis were done to investigate the thermal stability of both wild-type and mutant proteins. Melting curve analysis was performed at 222 nm. CD spectra were recorded at 30^{o}C/hr with a 0.1^{o}C step size, 2 s response time, and 1.0 nm bandwidth. Protein samples (10 μM) were prepared in HMK buffer (20 mM HEPES, 5 mM $MgCl_2$, and 100 mM KCl, pH 7).

3. RESULTS

According to our hypothesis, it was thought that there is a network between His226, Asp85, Thr225 and Arg71 residues which may have critical roles in the ATPase allostery. In this regard, to understand the role of His226, Asp85, Thr225, Arg71 and His295 residues in the ATPase allostery, a series of point mutations were generated in linkerless version of ATPase domain, DnaK(1-388). All point mutations were designed to investigate their effects on the pH-dependent ATPase activity and stability. Initially, His226, Thr225, Asp85 and Arg71 were mutated to alanine to prevent formation of any salt bridge interactions by the side chains. His226, in addition, is mutated to phenylalanine. Phenylalanine and histidine share a similar size but phenylalanine is unable to form a salt bridge, so with this replacement we aim to observe the effect of the size of histidine. On the other hand, Asp85 is also replaced by a glutamic acid to again understand its size effect in its interactions. His295, a distant site, was investigated for its possible effect on the pH-dependent ATPase activity. His295 was mutated to an aspartic acid which will cause an opposite effect due to the charge inversion.

3.1 Results of Mutagenesis Studies

All point mutations (H226A, H226F, H295D, T225A, D85A, D85E and R71A) in the *dnaK(1-388)* were created by Quick Change (Stratagene) Site-Directed mutagenesis kit. Based on site-directed mutagenesis method, mutagenesis PCR was performed using plasmid pMS-DnaK(1–388) as a template. After the mutagenesis PCR, the resultant PCR products were run on a 1% agarose gel and expected DNA fragments were detected. GeneRuler 1 kb Plus DNA Ladder, (75-20,000 bp) was used to estimate the PCR amplicon size. PCR fragments are shown in Figure 3.1. Following the mutagenesis PCR, a digestion step was performed using Dpn-I restriction enzyme in order to remove nonmutated parental supercoiled dsDNA template. After the digestion step, newly synthesized mutated plasmids were transformed into XL-1 Blue cells for the repair of nicked circular strands. In the

initial transformation studies, any colony was not observed although PCR amplicons were seen in the gel. Therefore, an additional purification step was applied for PCR products using QIAquick PCR Purification Kit. After the purification, transformation was repeated and colonies were obtained. Then, all plasmids containing specific mutations were isolated with QIAGEN, QIAPrep Spin Miniprep Kit and all isolated plasmids were checked on a 1% agarose gel.

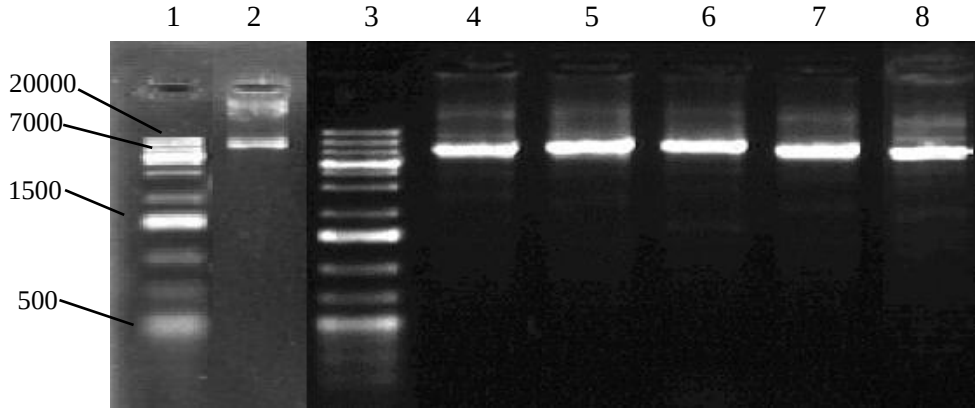

Figure 3.1 : 1% gel electrophoresis showing the result of mutagenesis PCR for *dnaK(1-388)* mutants. Lane 1 and 3: GeneRuler 1kb Plus DNA ladder, Lane 2: H226A PCR product, Lane 4: D85A PCR product, Lane 5: D85E PCR product, Lane 6: T225A PCR product, Lane 7: R71A PCR product, Lane 8: H295D PCR product.

Isolated plasmids are shown in Figure 3.2. After the plasmid isolation, presence of desired mutations was verified by sequencing. Analysis of sequence results was performed via EMBOSS Needle - Pairwise Sequence Alignment Tool. Sequencing results are given in Appendix D. Our results revealed that all the replacement mutations were successful.

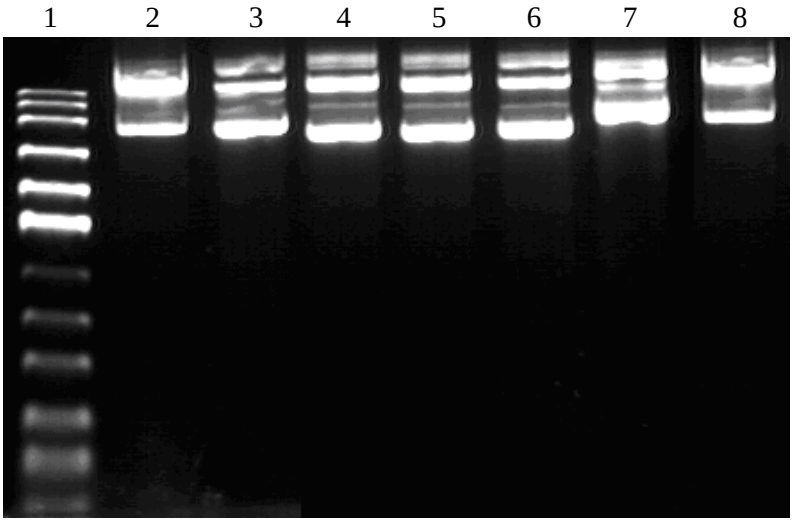

Figure 3.2 : 1% gel electrophoresis showing the all isolated plasmids containing *dnaK(1-388)* mutants. Lane 1: GeneRuler 1kb Plus DNA ladder, Lane 2: plasmid of H226A, Lane 4: plasmid of D85A, Lane 5: plasmid of D85E, Lane 6: plasmid of T225A, Lane 7: plasmid of R71A, Lane 8: plasmid of H295D.

3.2 Expression and Purification of DnaK(1-388) Proteins

Wild-type and mutated *dnaK* vectors were expressed in the *E.coli* BB1553 cells. Overexpression of DnaK(1-388) proteins were done by IPTG induction. Then, induction was analysed by SDS-PAGE. As shown in Figure 3.3 and Figure 3.4, wild-type and all mutant DnaK(1-388) proteins (42 kDa) could be overexpressed successfully.

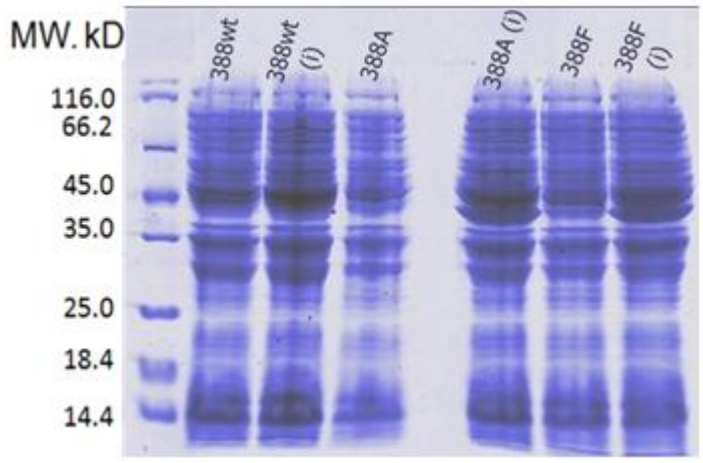

Figure 3.3 : SDS-PAGE analysis of total protein samples. Thick gel bands at about 45 kDa indicate the induction. (388wt: uninduced protein sample of DnaK(1-388) wild-type; 388wt(i): induced protein sample of DnaK(1-388) wild-type; 388A: uninduced protein sample of H226A; 388A(i): induced protein sample of H226A; 388F: uninduced protein sample of H226F; 388F(i): induced protein sample of H226F.

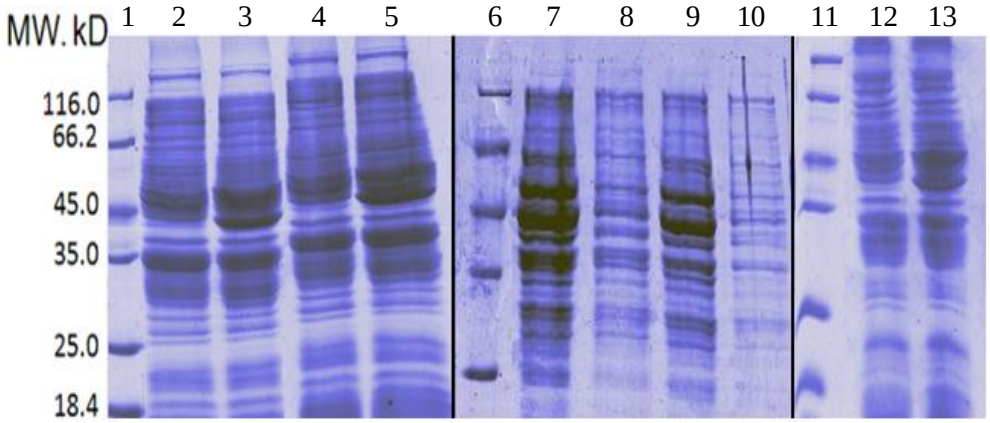

Figure 3.4 : SDS-PAGE analysis of total protein samples. Thick gel bands at about 45 kDa indicate the induction. Lane 1, 6, 11: Thermo Scientific Unstained Protein Molecular Weight Marker; Lane 2: uninduced protein sample of H295D; Lane 3: induced protein sample of H295D; Lane 4: uninduced protein sample of T225A; Lane 5: induced protein sample of T225A; Lane 7: induced protein sample of D85A; Lane 8: uninduced protein sample of D85A; Lane 9: induced protein sample of D85E; Lane 10: uninduced protein sample of D85E, Lane 12: uninduced protein sample of R71A; Lane 13: induced protein sample of R71A.

Corresponding mutant proteins were later isolated from BB1553(*ΔDnaK*) cells to a purity. To purify DnaK(1-388) proteins, DEAE–sephacel anion exchange and ATP-agarose column chromatography were used sequentially. Purified 42 kDa DnaK(1-388) wild-type and mutant proteins are shown in Figure 3.5.

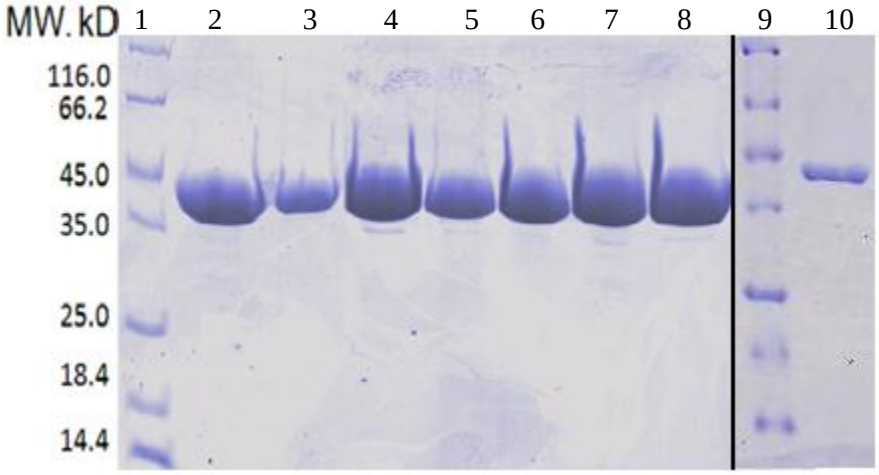

Figure 3.5 : Purified DnaK(1-388) wild-type and mutant proteins. (Lane 1 and 9: Thermo Scientific Unstained Protein Molecular Weight Marker (14.4 to 116 kDa), Lane 2: purified protein of DnaK(1-388) wild-type, Lane 3: purified protein of H226F, Lane 4: purified protein of T225A, Lane 5: purified protein of H226A, Lane 6: purified protein of H295D, Lane 7: purified protein of D85E, Lane 8: purified protein of D85A, Lane 10: purified protein of R71A.

3.3 Results of ATPase Assay

ATPase assays were performed between pH 5.5 and 8.5 to investigate the effects of mutations to the pH-activity profile. Initially, ATPase assay was performed for DnaK(1-388) wild-type to compare the activity results of our DnaK(1-388) mutants and also to reproduce previous results. The ATPase rate of DnaK(1-388) wild-type was measured at around 0.15 mol ATP.mol $DnaK^{-1}.min^{-1}$ at almost all pH values confirming the previous findings of Swain et al. for this construct (Swain et al., 2007).

When we compared H226A and H226F mutants with DnaK(1-388) wild-type, we found that H226A and H226F mutants led to a four and two-fold incease in the ATPase rate, respectively at almost all pH values (Figure 3.6). Although H226A showed slightly higher rates than that of H226F at all pH values, both of them showed similar pH-activity profile. In their pH activity profiles most striking pH value was pH 6 where an obvious hump was observed and this elevation was also observed for the wild-type with a small hump (Figure 3.6). In the pH-activity profile of H226A and H226F, it was also observed that ATPase activity increased toward pH 7.5 and 8. But this enhancement was not observed at pH 8.5 where wild-type like rate was detected.

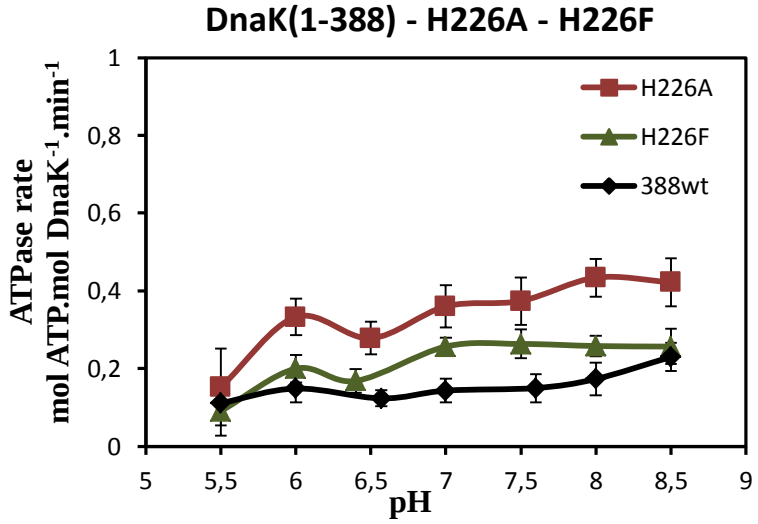

Figure 3.6 : ATPase rates of H226A and H226F compared to wild-type DnaK(1-388). Error bars represent standard deviation from three or more experiments.

T225A showed similar ATPase activity profile with H226A and H226F. The hump at pH 6 was also observed for this mutant (Figure 3.7). On the other hand a similar decrease was detected at pH 8.5.

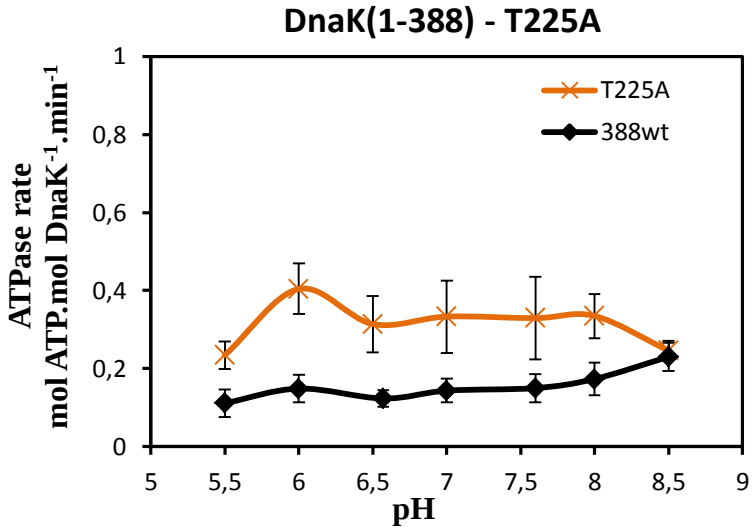

Figure 3.7 : ATPase rates of T225A compared to wild-type DnaK(1-388). Error bars represent standard deviation from three or more experiments.

Similar to the H226A and T225A mutants, H295D led to a threefold increase in the overall ATPase rate (Figure 3.8). But unlike the H226A and T225A mutants H295D did not show a hump at pH 6 and caused a small decrease at this pH.

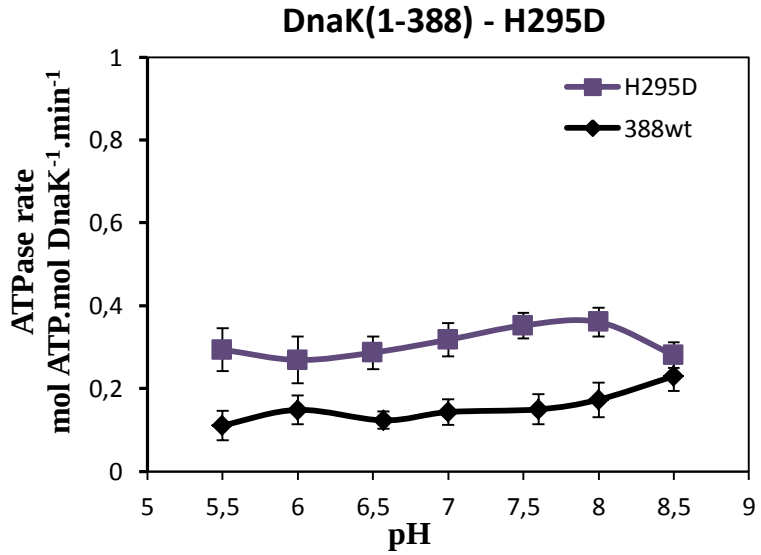

Figure 3.8 : ATPase rates of H295D compared to wild-type DnaK(1-388). Error bars represent standard deviation from three or more experiments.

When D85A was compared with the wild-type construct, a significant increase was observed in the overall ATPase rate. D85A caused 16-fold increase at almost all pH values. Unlike D85A, D85E showed similar ATPase rate with the wild-type construct. In the pH-activity profile of D85E, a small decrease was observed at pH 6 which was similar to H295D. In addition, this mutant showed a small hump at pH 7.5 (Figure 3.9). Previous study of Swain et al. and our current studies revealed a maximum activity at pH 7.5 for DnaK(1-392). So the observed shift in the pH-dependent ATPase activity profile of D85E and H295D from pH 6 to 7.5 suggests that these mutants act as if they are in the stimulated form and show a conformation similar to that of DnaK(1-392). But the magnitude of the activity was around twofold less for these mutants when compared to that of DnaK(1-392). It seems like these mutations can stimulate ATPase to a lesser extend than DnaK(1-392).

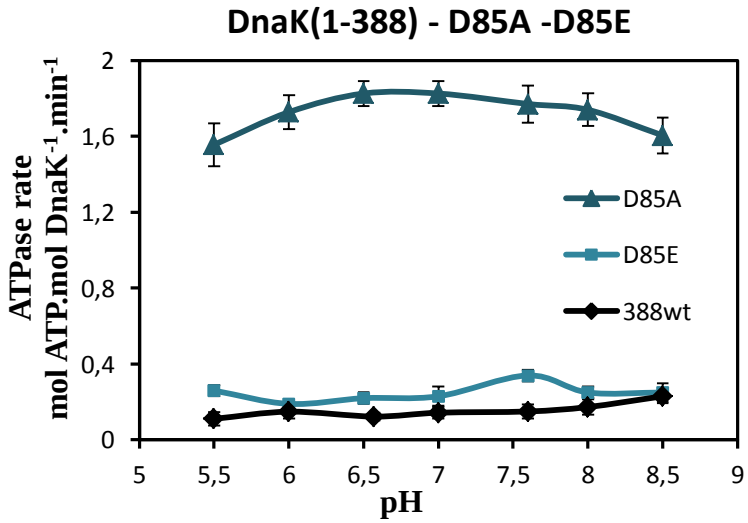

Figure 3.9 : ATPase rates of D85A and D85E compared to wild-type DnaK(1-388). Error bars represent standard deviation from three or more experiments.

Similar to D85A, R71A also caused 16-fold increase in the overall ATPase rate (Figure 3.10). The difference between these two mutants in the pH-activity profile was that R71A showed a hump at pH 7.5 while D85A showed similar ATPase rates at the pH range from 6 to 8. On the other hand, both of them showed a decrease in the ATPase rate at 8.5 (Figure 3.9 and 3.10). The decrease at this pH was observed for almost all measurements which can be derived from loss of enzymatic acitivty of the PK/LDH enzymes used in the ATPase assay.

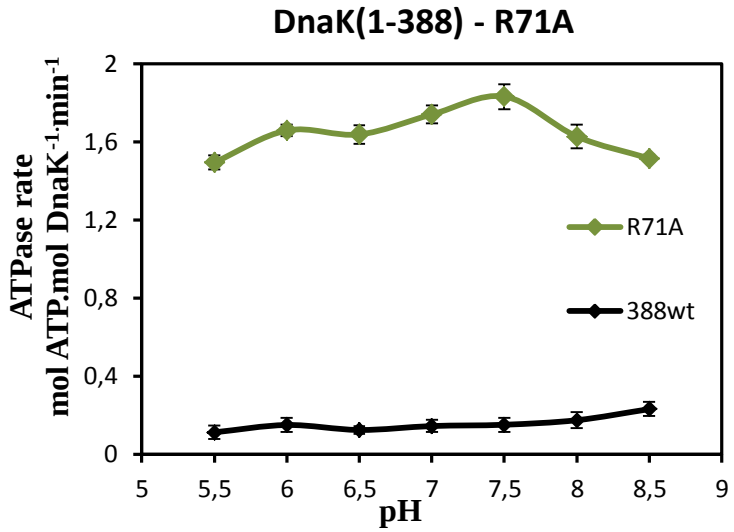

Figure 3.10 : ATPase rate of R71A compared to wild-type DnaK(1-388). Error bars represent standard deviation from three or more experiments.

3.4 Analysis of the Native Forms of DnaK(1-388) and Its Mutants

Native polyacrylamide gel electrophoresis was used to investigate the native forms of DnaK(1-388) wild-type and its mutants in the presence or absence of ATP. According to native gel results, wild-type construct appeared as a single band in both ATP presence and absence, indicating that wild-type construct did not lead to any oligomerization. H295D was run on the native gel with concentrations from 5 µM to 30 µM which is shown in Figure 3.11. This gel demonstrated the oligomerization pattern of H295D in the presence of ATP which was clearly observed at high concentrations (20 µM and 30 µM). This gel also showed that D85A did not cause any oligomerization and it migrated slower than wild-type and H295D. This case was also observed on another native gel where D85A was run on the gel as an increasing concentration, from 5µM to 30 µM (Figure 3.12). In contrast to D85A, native gel of D85E showed oligomerization. In this gel, distinctive polymer forms of D85E were clearly observed in the presence of ATP in the concentrations of 5 µM, 10 µM and 30 µM of D85E (Figure 3.13).

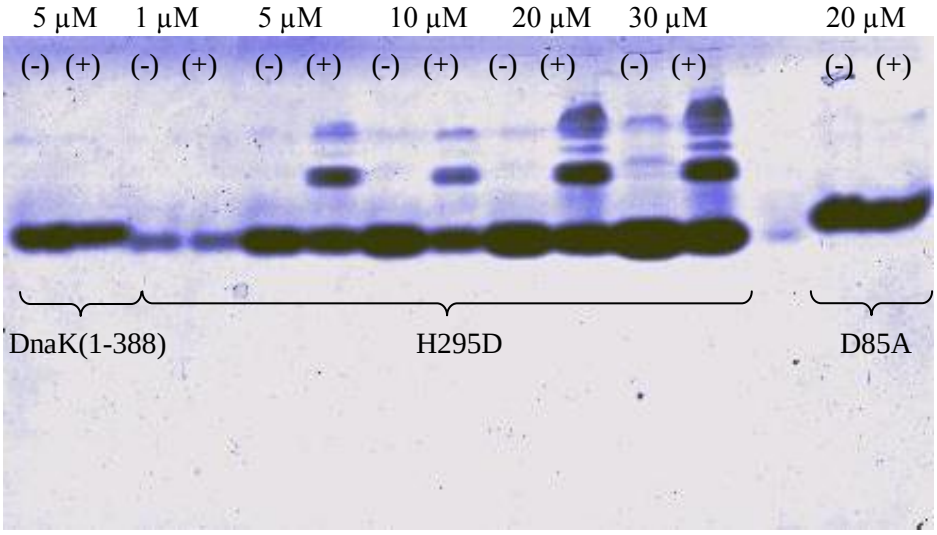

Figure 3.11 : DnaK(1-388) wild-type, H295D and D85A mutants on the native gel [(+): with ATP; (-): without ATP].

Oligomerization of D85E could not be clearly detected in the concentration of 20 µM that can be caused by mis or overloading of samples into the gel or by not mixing ATP properly in the samples. To verify the oligomerization differences between the presence and absence of ATP, D85E was run on another native gel which clearly demonstrated the oligomerization in the ATP-bound state (Figure 3.14).

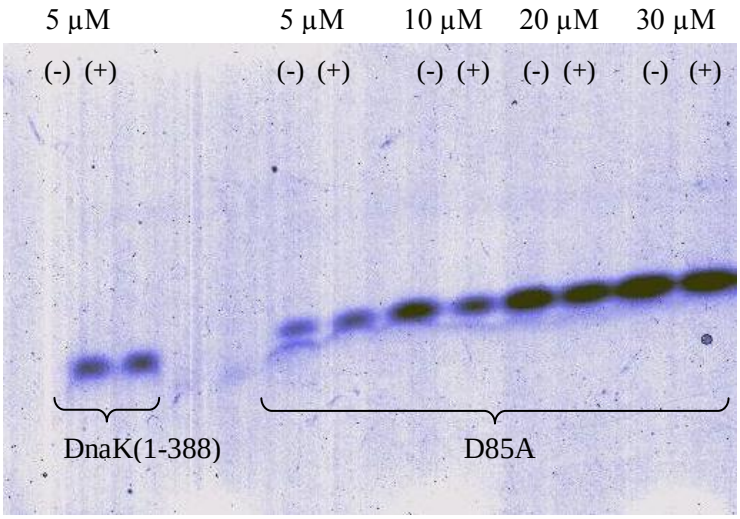

Figure 3.12 : DnaK(1-388) wild-type and D85A on the native gel [(+): with ATP; (-): without ATP].

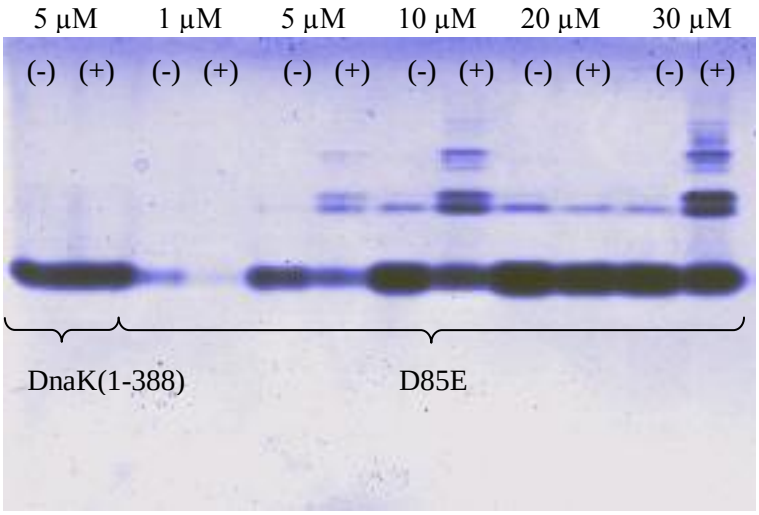

Figure 3.13 : DnaK(1-388) wild-type and D85E on the native gel [(+): with ATP; (-): without ATP].

A similar oligomerization with H295D and D85E was also observed for T225A. In the native gel showing the 10 µM and 20 µM concentration loadings of T225A, oligomerization for this mutant was clearly observed in the presence of ATP suggesting ATP-induced conformational rearrangements allows for polymer like structure formation for T225A (Figure 3.14). R71A mutant showed faint bands at higher molecular weights indicating presence of higher oligomeric forms of the protein (Figure 3.15). But unlike other mutants, this mutation revealed oligomeric forms both in the presence and absence of ATP suggesting ATP-induced conformational rearrangements do not allow a conformation for oligomerization.

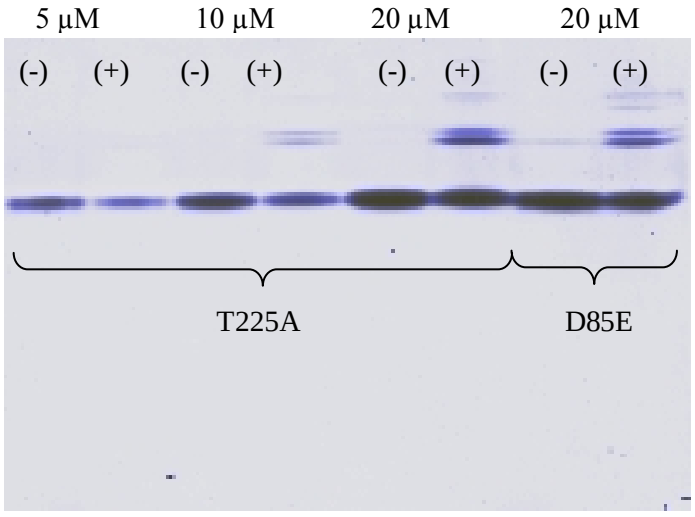

Figure 3.14 : T225A and D85E on the native gel [(+): with ATP; (-): without ATP].

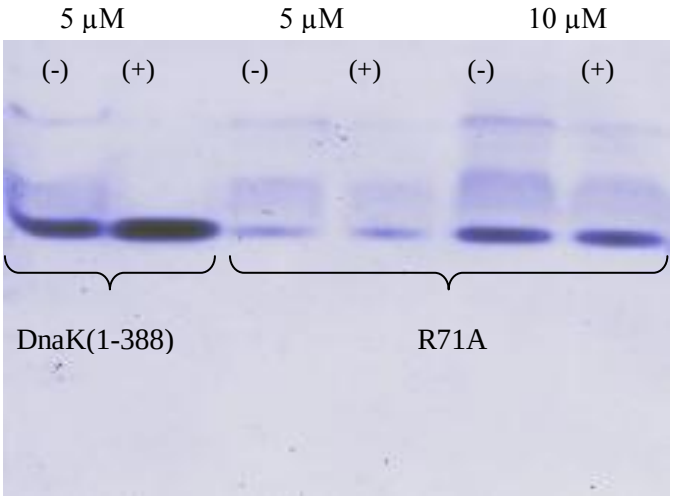

Figure 3.15 : DnaK(1-388) wild-type and R71A on the native gel [(+): with ATP; (-): without ATP].

On the other hand, native gel of the His226 mutants demonstrated that H226A and H226F did not lead to any oligomerization (Figure 3.16). In addition we did not observe any differences in their mobilities.

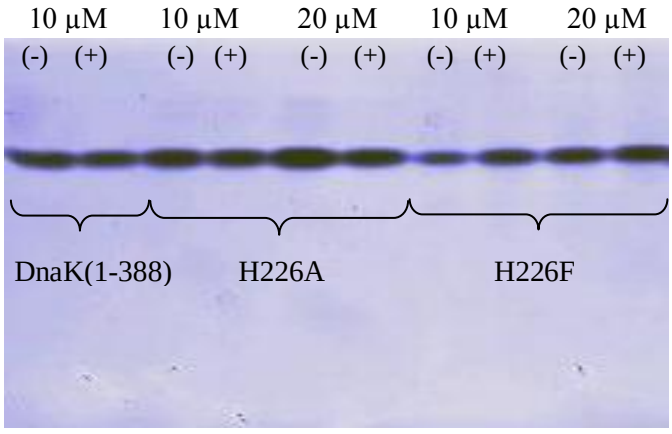

Figure 3.16 : DnaK(1-388) wild-type, H226A and H226F on the native gel [(+): with ATP; (-): without ATP].

3.5 Secondary Structure Analyses of DnaK(1-388)wt and Its Mutants

Secondary structural features of both wild-type and mutant proteins were analyzed using circular dichroism spectroscopy. CD scans were performed in wavelength range of 190 to 250 nm and these scans were repeated at least three times. CD spectra for DnaK(1-388) wild-type and H226A, H226F mutants are shown in Figure 3.17. These spectra demonstrated that there was no obvious change in the secondary structure of H226A and H226F mutants and wild-type construct.

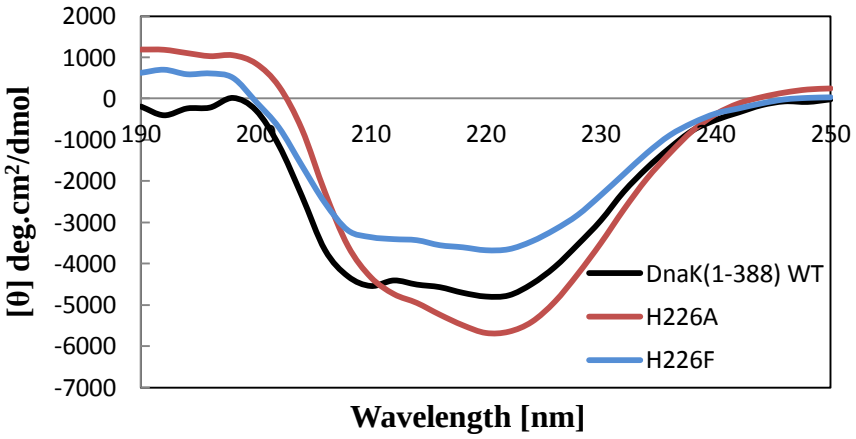

Figure 3.17 : Circular dichroism spectra of DnaK(1-388)wt, H226A, H226F and D85A.

3.6 Stability of DnaK(1-388)wt and Its Mutants

Melting curve analyses were performed to investigate thermal stability of both wild-type and mutant proteins at pH 7 using circular dichroism spectroscopy. All measurements were performed using 10 µM protein samples.

Melting curve analysis were carried out for DnaK(1-388) wild-type, H226A and H226F (Figure 3.18). Transition midpoint (T_m) for DnaK(1-388) wild-type was found at around 51^{o}C. However, T_m value for H226A and H226F mutants were found at around 49^o indicating that these mutants decreased the stability on the ATPase domain.

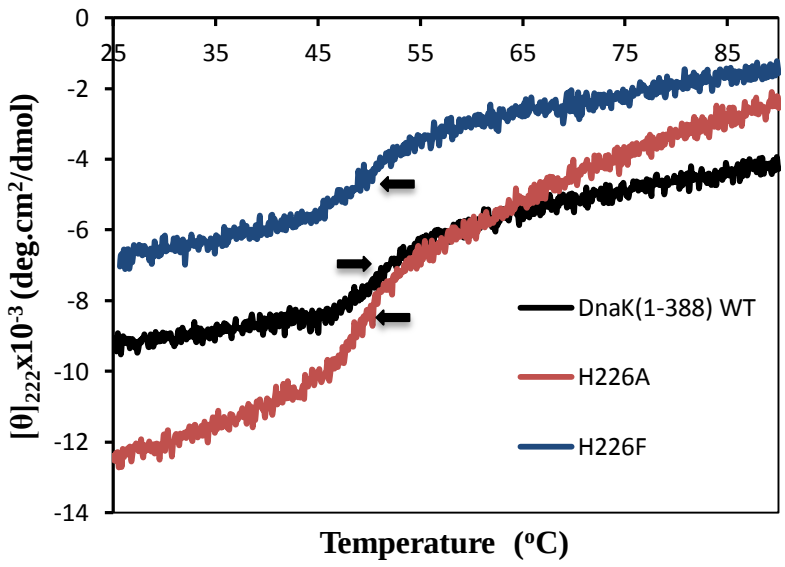

Figure 3.18 : Melting curve of DnaK(1-388)wt, H226A and H226F. Analysis was monitored by circular dichroism at 222 nm. Arrows in black show the thermal midpoint

4. DISCUSSION

Data from Swain et al. (Swain et al., 2007), Vogel et al. (Vogel et al., 2006) and recent findings of Zhuravleva et al (Zhuravlera et al., 2011) confirmed that DnaK(1-392) construct can be a model protein of Hsp70 that is mimicking the substrate stimulated form of the full-length protein. Linkerless version of the ATPase domain will be a useful construct to study ATPase domain dynamics in the unstimulated state of the domain, DnaK(1-388), thus, will be used in this study. Based on the crystal structure of ATPase domain we first suggested that there might be a network formed by interactions among His226, Asp85, Thr225 and Arg71 residues which may have critical roles in the ATPase allostery. In the light of this hypothesis, mutants of DnaK(1-388) were generated to delineate the sites that are critical for ATPase allostery in the ATPase domain, and mutations' functional and structural effects to the domain were studied using various biochemical and biophysical techniques.

It is known that full-length DnaK and its substrate stimulated form show different ATPase activity profiles as a function of pH (Swain et al., 2007). While substrate-stimulated form shows a pH dependent activity with a bell shaped profile having maximum at pH 7.5, the unstimulated form shows almost a steady activity as a function of pH with a rate at around 0.15 mol ATP.mol $DnaK^{-1}.min^{-1}$. A similar variation is observed in the activity of the ATPase domain constructs: DnaK(1-392) showed a pH dependent activity, like the stimulated form of the full-length DnaK; on the other hand, DnaK(1-388) showed a similar activity profile as like the unstimulated form of full-length DnaK (Swain et al., 2007). We first tested the activity of wild-type DnaK(1-388), and succesfully reproduced the earlier findings. When we later compared our DnaK(1-388) mutant activity results with the wild-type construct, we observed some dramatic changes caused by mutations. Thr225 and His226 replacements showed a similar effect to the activity. Both mutations showed subtle elevation in the rate for the measured range of pH. The observed weak inclination at pH 6.0 for the wild-type could be detected as an obvious hump for the

Thr 225 and His 226 mutants. The rest of the pH activity profiles of these mutants seem to be correlating well, just by a twofold enhancement in the rate, with the wild-type ATPase domain construct. Growing a peak at around pH 6.0 reveals that for wild-type and mutant constructs there are titratiable residues around this pH in the activated form. Interestingly, the ATPase domain construct containing the linker does not show a peak around that pH, rather it reveals a peak at pH 7.6. Therefore, this study revealed the difference in the activity mechanisms of the ATPase domain when linker is present or absent. Results of a fluorescence study involving the measurement of the ADP release rate from Swain et al. (Swain et al., 2007) revealed that ADP release is the rate limiting step of the ATPase domain when linker induces the rate. Our findings indicate clearly ATPase activation mechanism differences as a function of pH, thus for the mutants causing an alteration in the ATPase mechanism can be evaluated by comparing the pH-dependent ATPase activity profiles with these constructs. When pH-activity profiles of Thr225 and His226 replacements were compared to that of the ATPase constructs, mutations of these sites did not alter the ATPase mechanism.

Mutations on the Asp 85 residue showed that this residue plays an important role in the modulation of ATPase activity. Replacement of Asp85 residue with alanine amino acid caused a 16 fold increase in the ATPase rate. But it was seen that this enhancement was not pH sensitive. Chang et al. study revealed that D85A mutation in the full-length DnaK causes an increase in the basal ATPase activity, although it disrupts the substrate stimulation (Chang et al., 2010). This result supports our results. Furthermore, results of Chang et al. study and our study support the view that Asp85 has a repressive effect on the modulation of the ATPase activity. Present study showed that replacement of Asp85 residue with a glutamic acid did not cause any increase in the ATPase activity. Based on this data, it can be said that negative charge of the aspartic acid is significant for this repsessive effect. In addition to these ATPase assay results, native gels demonstrated that D85A mutation causes slow migration than wild-type construct while D85E showed same mobility with wild-type construct. These results can be explained with a view that loss of the negative charge makes significant differences in the conformation of DnaK(1-388) and these conformational alterations affects the mechanism of ATPase.

Mutation of His 295 to Ala which is located at a distant side from the nucleotide binding cleft caused a twofold enhancement in the ATPase rate of DnaK(1-388) at all measured pH values. When we compared H295D mutation with H226A and T225A in terms of ATPase activity, the hump at pH 6 was not detected for H295D mutation. This result indicated that His295 affects the mechanism of ATPase activity in a different way as His226 and Thr225 did. Native gel results demonstated that in contrast to wild-type construct, H295D and D85E mutations lead to oligomerization in the ATP-bound state. This observation suggest that in the ATP-free state, linker binding region (the hydrophobic cleft between subdomains IA and IIA of ATPase domain) of wild-type DnaK(1-388) is closed while in the ATP-bound state, this region is exposed, thus, negatively charged aspartic acid in the H295D mutation or glutamic acid in the D85E mutation may interact with this exposed region seeking a negative charge, thus leading to a oligomerization in the ATP-bound state. On the other hand, oligomerization of the D85E also indicated that size differences between aspartic acid and glutamic acid is crucial for the interactions with the exposed region.

Our data demonstrated that similar polymer forms occur for D85E and T225A mutants in the ATP-bound state. In contrast, oligomerization was not observed for D85A. On the other hand, we found that D85A increased the ATPase activity by 16 fold while D85E and T225A could not increase the rate as D85A did. From our results, we think that there is an interaction between Thr225 and Asp85 residues. Replacement of Asp85 by alanine causes a loss of negative charge and that is why we did not observe poylmerization for D85A. As we suggested negative charge is important for oligomerization and in this case alanine can not interact with exposed linker binding region and so can not allow oligomerization. Loss of negative charge can disrupt the interaction between Thr225 and Asp85. Conformational alterations caused by D85A mutation may affect the positioning of K70 which is the catalytic residue in the ATPase domain and thus, causes significant increase in the ATPase rate. Replacement of Thr225 by alanine can also disrupt the interaction between Thr225 and Asp85. In this case we observed the oligomerization because negative charge of the Asp85 is exposed and allows for oligomerization.

Replacement of Arg71 caused a dramatic change in the ATPase rate which increased the rate by 16-fold as D85A did. This significant increase demonstrated that Arg71 also plays a crucial role in the modulation of ATP hydrolysis like Asp85. However,

considering the pH-activity profile of R71A which showed a hump at pH 7.5 unlike D85A, it can be said that Arg71 affects the ATP hydrolysis in a different way. But, according to these results it is not possible to explain how Arg71 and Asp85 residues affect the modulation of ATP hydrolysis rate.

In conclusion, this study revealed that the activity mechanism of the ATPase domain is different for linkerless version, DnaK(1-388), compared to DnaK(1-392). This difference was revealed by pH activity profile of H226A, H226F and T225A mutants. In addition, we observed oligomerization for some mutants of DnaK(1-388) suggesting that the hydrophobic cleft between subdomains IA and IIA of ATPase domain is exposed and interact with negative residues while we did not observed oligomerization for any mutants of DnaK(1-392) (data not shown) suggesting that this cleft has already been occupied by the linker. On the other hand, we found the repressive effects of the Asp85 and Arg71 residues on the modulation of the ATPase activity in the linkerless version, DnaK(1-388) and we also found possible interactions between Asp85 and Thr225 residues. But further studies are needed to understand how these residues affect the dynamics of the ATPase domain.

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APPENDICES

APPENDIX A: Laboratory equipments

APPENDIX B: Chemicals and enzymes

APPENDIX C: Preparation of buffer and solutions

APPENDIX D: Sequencing results

APPENDIX A:

Table A.1: Laboratory equipments used in the study

Equipment	**Supplier Company**
Electrophoresis Gel System	Cleaver Scientific
Power supply	GE Healthcare
Centrifuges	Beckman Coulter Avanti TM J-30 I, IECCL10 Centrifuge
Thermal Cycler	Techne TC-3000
UV Transilluminator	Biorad UV Transilluminator 2000 Vilber Lourmat
UVIPhoto MW Version 99.05 for Windows 95 & 98	UVItec Ltd.
FPLC	Biorad Biologic DuoFlow
FPLC Columns	Biorad Econo-Column
SDS-PAGE gel electrophoresis system	Amersham Biosciences
pH Meter	Mettler Toledo
Magnetic stirrer	IKAMAG
Vortex	Heidolph, Reax top
Laminar air flow cabinets	FASTER BH-EN 2003
Freezers	New Brunswick Scientific (-80^{o}C)
Pure Water System	UHQ USF Elga TKA Wasseraufbereitungssysteme
Ice machine	Scotsman AF 10
Shaker	Forma Orbital Shaker, Thermo Electron Corporation
UV-Visible Spectrophotometer	Thermo Scientific NanoDrop 2000
Microplate Spectrophotometer	Biorad Benchmark Plus
Precision Weigher	Precisa 620C SCS
Weigher	Precisa BJ 610 C
Pipettes	Gilson

APPENDIX B:

Table B.1 : Chemicals and enzymes used in the study

Chemicals and enzymes	**Supplier Company**
Yeast Extract	Merck
Agar	Merck
NaCl	Merck
MgCl	Merck
CaCl	Merck
Mg(CoA)	Merck
Pepton	Fluka
SDS	Molecula
EDTA	Molecula
APS	Molecula
Tris	ABCR
DEAE Sephacel	GE Healtcare
ATP agarose	Sigma
PEP	Sigma
ATP	Sigma
NADH	Sigma
PK/LDH enzyme coctail	Sigma
Chloramphenicol	Sigma
Ampicillin	Roth
SYBR Green	Roche
Laemmli protein sample buffer	Sigma
Protein marker	PeqLab
IPTG	PeqLab
Agarose	PeqLab
Acrylamid Solution	Biorad
PMSF	Fluka
Leupeptin	Roth
Pepstain A	Roth
Lysosyme	Roth

APPENDIX C:

Luria Bertani (LB) medium was prepared by dissolving 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 L dH_2O. The media wassterilized by autoclaving at 121^oC for 10 minutes. After sterilization, in order to make selection, appropriate antibiotics (ampicillin - chloramphenicol) were added. To prepare LB Agar Medium**,** additional 20 g Agar was added to 1L LB medium**.**

Resuspension buffer was used in the growth step of BB1553 cells**.** To prepare resuspension buffer, 20 mM Tris, 50 mM NaCl, 0.1 mM EDTA were dissolved in 1 L dH_2O and pH is adjusted to 7.4. Then, it was filtered by 0.2 µm for sterilization.

Buffer A was used in the first step of DnaK Purification by DEAE–Sephacel column. To prepare Buffer A, 20 mM Tris-HCl, 1 mM EDTA were dissolved in 1 L dH_2O and pH is adjusted to 7.4. Then, it was filtered by 0.2 µm filter for sterilization.

Buffer B was also in the first step of DnaK Purification by DEAE–Sephacel column. To prepare Buffer B, 20 mM Tris-HCl, 1 mM EDTA and 1 M NaCl were dissolved in 1 L dH_2O and pH is adjusted to 7.4. Then, it was filtered by 0.2 µm filter for sterilization.

HMK buffer was used in the second step of DnaK Purification by ATP-agarose column. To prepare HMK buffer, 20 mm HEPES, 5 mm $MgCl_2$ and 100 mm KCl were dissolved in 1 L dH_2O and pH is adjusted to 7.4. Then, it was filtered by 0.2 µm filter for sterilization.

HEK buffer was also used in the second step of DnaK Purification by ATP-agarose column. To prepare HEK buffer, 20 mm HEPES, 10 mm EDTA and 100 mm KCl were dissolved in 1 L dH_2O and pH is adjusted to 7.4. Then, it was filtered by 0.2 µm filter for sterilization.

Running buffer for SDS-PAGE analysis: Tris-Glycine SDS Buffer (10X) is used as the electrophoresis running buffer during the stacking and resolve process of SDS-PAGE. To prepare 10X running buffer, 30.3 g Tris, 144 g glycine and 10 g SDS were dissolved in 1L with dH_2O. 10X SDS buffer was used by diluting to 1X SDS buffer with dH_2O.

Coomassie Brilliant Blue (CBB) stain solution: 0.5 g CBB R-250 was mixed with 250 ml methanol and 50 ml acetic acid and up to 500 ml with dH_2O.

Destain solution: 450 ml methanol and 50 ml acetic acid were mixed and up to 1 L with dH_2O.

APPENDIX D:

DnaK(1-388) H226A

```
(1-388)H226A       1 ----------------------ACGAAGTTGACGGCGAAAAAACCTTCGA     28
                                            ||||||||||||||||||||||||||||
wtDnaK           601 GATATTTCTATTATCGAAATCGACGAAGTTGACGGCGAAAAAACCTTCGA    650

(1-388)H226A      29 AGTTCTGGCAACCAACGGTGATACCGCCCTGGGGGGTGAAGACTTCGACA     78
                     ||||||||||||||||||||||||||..||||||||||||||||||||||||
wtDnaK           651 AGTTCTGGCAACCAACGGTGATACCCACCTGGGGGGTGAAGACTTCGACA    700

(1-388)H226A      79 GCCGTCTGATCAACTATCTGGTTGAAGAATTCAAGAAAGATCAGGGCATT    128
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           701 GCCGTCTGATCAACTATCTGGTTGAAGAATTCAAGAAAGATCAGGGCATT    750

(1-388)H226A     129 GACCTGCGCAACGATCCGCTGGCAATGCAGCGCCTGAAAGAAGCGGCAGA    178
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           751 GACCTGCGCAACGATCCGCTGGCAATGCAGCGCCTGAAAGAAGCGGCAGA    800

(1-388)H226A     179 AAAAGCGAAAATCGAACTGTCTTCCGCTCAGCAGACCGACGTTAACCTGC    228
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           801 AAAAGCGAAAATCGAACTGTCTTCCGCTCAGCAGACCGACGTTAACCTGC    850

(1-388)H226A     229 CATACATCACTGCAGACGCGACCGGTCCGAAACACATGAACATCAAAGTG    278
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           851 CATACATCACTGCAGACGCGACCGGTCCGAAACACATGAACATCAAAGTG    900

(1-388)H226A     279 ACTCGTGCGAAACTGGAAAGCCTGGTTGAAGATCTGGTAAACCGTTCCAT    328
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           901 ACTCGTGCGAAACTGGAAAGCCTGGTTGAAGATCTGGTAAACCGTTCCAT    950

(1-388)H226A     329 TGAGCCGCTGAAAGTTGCACTGCAGGACGCTGGCCTGTCCGTATCTGATA    378
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           951 TGAGCCGCTGAAAGTTGCACTGCAGGACGCTGGCCTGTCCGTATCTGATA   1000

(1-388)H226A     379 TCGACGACGTTATCCTCGTTGGTGGTCAGACTCGTATGCCAATGGTTCAG    428
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK          1001 TCGACGACGTTATCCTCGTTGGTGGTCAGACTCGTATGCCAATGGTTCAG   1050

(1-388)H226A     429 AAGAAAGTTGCTGAGTTCTTTGGTAAAGAGCCGCGTAAAGACGTTAACCC    478
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK          1051 AAGAAAGTTGCTGAGTTCTTTGGTAAAGAGCCGCGTAAAGACGTTAACCC   1100

(1-388)H226A     479 GGACGAAGCTGTAGCAATCGGTGCTGCTGTTCAGGGTGGTGTTCTGACTG    528
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK          1101 GGACGAAGCTGTAGCAATCGGTGCTGCTGTTCAGGGTGGTGTTCTGACTG   1150
```

DnaK(1-388) H226F

```
(1-388)H226F       1 ----------------------ACGAAGTTGACGGCGAAAAAACCTTCGA     28
                                            ||||||||||||||||||||||||||||
wtDnaK           601 GATATTTCTATTATCGAAATCGACGAAGTTGACGGCGAAAAAACCTTCGA    650

(1-388)H226F      29 AGTTCTGGCAACCAACGGTGATACCTTCCTGGGGGGTGAAGACTTCGACA     78
                     ||||||||||||||||||||||||||..||||||||||||||||||||||||
wtDnaK           651 AGTTCTGGCAACCAACGGTGATACCCACCTGGGGGGTGAAGACTTCGACA    700

(1-388)H226F      79 GCCGTCTGATCAACTATCTGGTTGAAGAATTCAAGAAAGATCAGGGCATT    128
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           701 GCCGTCTGATCAACTATCTGGTTGAAGAATTCAAGAAAGATCAGGGCATT    750

(1-388)H226F     129 GACCTGCGCAACGATCCGCTGGCAATGCAGCGCCTGAAAGAAGCGGCAGA    178
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           751 GACCTGCGCAACGATCCGCTGGCAATGCAGCGCCTGAAAGAAGCGGCAGA    800

(1-388)H226F     179 AAAAGCGAAAATCGAACTGTCTTCCGCTCAGCAGACCGACGTTAACCTGC    228
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           801 AAAAGCGAAAATCGAACTGTCTTCCGCTCAGCAGACCGACGTTAACCTGC    850

(1-388)H226F     229 CATACATCACTGCAGACGCGACCGGTCCGAAACACATGAACATCAAAGTG    278
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           851 CATACATCACTGCAGACGCGACCGGTCCGAAACACATGAACATCAAAGTG    900
```

```
(1-388)H226F     279 ACTCGTGCGAAACTGGAAAGCCTGGTTGAAGATCTGGTAAACCGTTCCAT    328
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           901 ACTCGTGCGAAACTGGAAAGCCTGGTTGAAGATCTGGTAAACCGTTCCAT    950

(1-388)H226F     329 TGAGCCGCTGAAAGTTGCACTGCAGGACGCTGGCCTGTCCGTATCTGATA    378
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           951 TGAGCCGCTGAAAGTTGCACTGCAGGACGCTGGCCTGTCCGTATCTGATA   1000

(1-388)H226F     379 TCGACGACGTTATCCTCGTTGGTGGTCAGACTCGTATGCCAATGGTTCAG    428
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK          1001 TCGACGACGTTATCCTCGTTGGTGGTCAGACTCGTATGCCAATGGTTCAG   1050

(1-388)H226F     429 AAGAAAGTTGCTGAGTTCTTTGGTAAAGAGCCGCGTAAAGACGTTAACCC    478
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK          1051 AAGAAAGTTGCTGAGTTCTTTGGTAAAGAGCCGCGTAAAGACGTTAACCC   1100

(1-388)H226F     479 GGACGAAGCTGTAGCAATCGGTGCTGCTGTTCAGGGTGGTGTTCTGACTG    528
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK          1101 GGACGAAGCTGTAGCAATCGGTGCTGCTGTTCAGGGTGGTGTTCTGACTG   1150
```

DnaK(1-388) D85A

```
(1-388)D85A        1 ----------------------------------------------TGTT      4
                                                                   ||||
wtDnaK           151 GGTCAGCCGGCTAAACGTCAGGCAGTGACGAACCCGCAAAACACTCTGTT    200

(1-388)D85A        5 TGCGATTAAACGCCTGATTGGTCGCCGCTTCCAGGACGAAGAAGTACAGC     54
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           201 TGCGATTAAACGCCTGATTGGTCGCCGCTTCCAGGACGAAGAAGTACAGC    250

(1-388)D85A       55 GTGCTGTTTCCATCATGCCGTTCAAAATTATTGCTGCTGATAACGGCGAC    104
                     |||.||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           251 GTGATGTTTCCATCATGCCGTTCAAAATTATTGCTGCTGATAACGGCGAC    300

(1-388)D85A      105 GCATGGGTCGAAGTTAAAGGCCAGAAAATGGCACCGCCGCAGATTTCTGC    154
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           301 GCATGGGTCGAAGTTAAAGGCCAGAAAATGGCACCGCCGCAGATTTCTGC    350

(1-388)D85A      155 TGAAGTGCTGAAAAAAATGAAGAAAACCGCTGAAGATTACCTGGGTGAAC    204
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           351 TGAAGTGCTGAAAAAAATGAAGAAAACCGCTGAAGATTACCTGGGTGAAC    400

(1-388)D85A      205 CGGTAACTGAAGCTGTTATCACCGTACCGGCATACTTTAACGATGCTCAG    254
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           401 CGGTAACTGAAGCTGTTATCACCGTACCGGCATACTTTAACGATGCTCAG    450

(1-388)D85A      255 CGTCAGGCAACCAAAGACGCAGGCCGTATCGCTGGTCTGGAAGTAAAACG    304
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           451 CGTCAGGCAACCAAAGACGCAGGCCGTATCGCTGGTCTGGAAGTAAAACG    500

(1-388)D85A      305 TATCATCAACGAACCGACCGCAGCTGCGCTGGCTTACGGTCTGGACAAAG    354
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           501 TATCATCAACGAACCGACCGCAGCTGCGCTGGCTTACGGTCTGGACAAAG    550

(1-388)D85A      355 GCACTGGCAACCGTACTATCGCGGTTTATGACCTGGGTGGTGGTACTTTC    404
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           551 GCACTGGCAACCGTACTATCGCGGTTTATGACCTGGGTGGTGGTACTTTC    600

(1-388)D85A      405 GATATTTCTATTATCGAAATCGACGAAGTTGACGGCGAAAAAACCTTCGA    454
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           601 GATATTTCTATTATCGAAATCGACGAAGTTGACGGCGAAAAAACCTTCGA    650

(1-388)D85A      455 AGTTCTGGCAACCAACGGTGATACCCACCTGGGGGGTGAAGACTTCGACA    504
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           651 AGTTCTGGCAACCAACGGTGATACCCACCTGGGGGGTGAAGACTTCGACA    700

(1-388)D85A      505 GCCGTCTGATCAACTATCTGGTTGAAGAATTCAAGAAAGATCAGGGCATT    554
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           701 GCCGTCTGATCAACTATCTGGTTGAAGAATTCAAGAAAGATCAGGGCATT    750
```

DnaK(1-388) D85E

```
(1-388)D85E        1 ------------------------------------------------TGTT      4
                                                                   ||||
wtDnaK           151 GGTCAGCCGGCTAAACGTCAGGCAGTGACGAACCCGCAAAACACTCTGTT    200

(1-388)D85E        5 TGCGATTAAACGCCTGATTGGTCGCCGCTTCCAGGACGAAGAAGTACAGC     54
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           201 TGCGATTAAACGCCTGATTGGTCGCCGCTTCCAGGACGAAGAAGTACAGC    250

(1-388)D85E       55 GTGAGGTTTCCATCATGCCGTTCAAAATTATTGCTGCTGATAACGGCGAC    104
                     ||||.||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           251 GTGATGTTTCCATCATGCCGTTCAAAATTATTGCTGCTGATAACGGCGAC    300

(1-388)D85E      105 GCATGGGTCGAAGTTAAAGGCCAGAAAATGGCACCGCCGCAGATTTCTGC    154
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           301 GCATGGGTCGAAGTTAAAGGCCAGAAAATGGCACCGCCGCAGATTTCTGC    350

(1-388)D85E      155 TGAAGTGCTGAAAAAAATGAAGAAAACCGCTGAAGATTACCTGGGTGAAC    204
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           351 TGAAGTGCTGAAAAAAATGAAGAAAACCGCTGAAGATTACCTGGGTGAAC    400

(1-388)D85E      205 CGGTAACTGAAGCTGTTATCACCGTACCGGCATACTTTAACGATGCTCAG    254
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           401 CGGTAACTGAAGCTGTTATCACCGTACCGGCATACTTTAACGATGCTCAG    450

(1-388)D85E      255 CGTCAGGCAACCAAAGACGCAGGCCGTATCGCTGGTCTGGAAGTAAAACG    304
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           451 CGTCAGGCAACCAAAGACGCAGGCCGTATCGCTGGTCTGGAAGTAAAACG    500

(1-388)D85E      305 TATCATCAACGAACCGACCGCAGCTGCGCTGGCTTACGGTCTGGACAAAG    354
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           501 TATCATCAACGAACCGACCGCAGCTGCGCTGGCTTACGGTCTGGACAAAG    550

(1-388)D85E      355 GCACTGGCAACCGTACTATCGCGGTTTATGACCTGGGTGGTGGTACTTTC    404
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           551 GCACTGGCAACCGTACTATCGCGGTTTATGACCTGGGTGGTGGTACTTTC    600

(1-388)D85E      405 GATATTTCTATTATCGAAATCGACGAAGTTGACGGCGAAAAAACCTTCGA    454
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           601 GATATTTCTATTATCGAAATCGACGAAGTTGACGGCGAAAAAACCTTCGA    650

(1-388)D85E      455 AGTTCTGGCAACCAACGGTGATACCCACCTGGGGGGTGAAGACTTCGACA    504
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           651 AGTTCTGGCAACCAACGGTGATACCCACCTGGGGGGTGAAGACTTCGACA    700

(1-388)D85E      505 GCCGTCTGATCAACTATCTGGTTGAAGAATTCAAGAAAGATCAGGGCATT    554
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           701 GCCGTCTGATCAACTATCTGGTTGAAGAATTCAAGAAAGATCAGGGCATT    750
```

DnaK(1-388) R71A

```
(1-388)R71A        1 ------------------------------------------------GATC      4
                                                                   ||||
wtDnaK            51 GATTATGGATGGCACCACTCCTCGCGTGCTGGAGAACGCCGAAGGCGATC    100

(1-388)R71A        5 GCACCACGCCTTCTATCATTGCCTATACCCAGGATGGTGAAACTCTAGTT     54
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           101 GCACCACGCCTTCTATCATTGCCTATACCCAGGATGGTGAAACTCTAGTT    150

(1-388)R71A       55 GGTCAGCCGGCTAAACGTCAGGCAGTGACGAACCCGCAAAACACTCTGTT    104
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           151 GGTCAGCCGGCTAAACGTCAGGCAGTGACGAACCCGCAAAACACTCTGTT    200

(1-388)R71A      105 TGCGATTAAAGCCCTGATTGGTCGCCGCTTCCAGGACGAAGAAGTACAGC    154
                     ||||||||||..||||||||||||||||||||||||||||||||||||||||
wtDnaK           201 TGCGATTAAACGCCTGATTGGTCGCCGCTTCCAGGACGAAGAAGTACAGC    250

(1-388)R71A      155 GTGATGTTTCCATCATGCCGTTCAAAATTATTGCTGCTGATAACGGCGAC    204
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           251 GTGATGTTTCCATCATGCCGTTCAAAATTATTGCTGCTGATAACGGCGAC    300

(1-388)R71A      205 GCATGGGTCGAAGTTAAAGGCCAGAAAATGGCACCGCCGCAGATTTCTGC    254
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           301 GCATGGGTCGAAGTTAAAGGCCAGAAAATGGCACCGCCGCAGATTTCTGC    350
```

```
(1-388)R71A      255 TGAAGTGCTGAAAAAAATGAAGAAAACCGCTGAAGATTACCTGGGTGAAC    304
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           351 TGAAGTGCTGAAAAAAATGAAGAAAACCGCTGAAGATTACCTGGGTGAAC    400

(1-388)R71A      305 CGGTAACTGAAGCTGTTATCACCGTACCGGCATACTTTAACGATGCTCAG    354
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           401 CGGTAACTGAAGCTGTTATCACCGTACCGGCATACTTTAACGATGCTCAG    450

(1-388)R71A      355 CGTCAGGCAACCAAAGACGCAGGCCGTATCGCTGGTCTGGAAGTAAAACG    404
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           451 CGTCAGGCAACCAAAGACGCAGGCCGTATCGCTGGTCTGGAAGTAAAACG    500

(1-388)R71A      405 TATCATCAACGAACCGACCGCAGCTGCGCTGGCTTACGGTCTGGACAAAG    454
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           501 TATCATCAACGAACCGACCGCAGCTGCGCTGGCTTACGGTCTGGACAAAG    550

(1-388)R71A      455 GCACTGGCAACCGTACTATCGCGGTTTATGACCTGGGTGGTGGTACTTTC    504
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           551 GCACTGGCAACCGTACTATCGCGGTTTATGACCTGGGTGGTGGTACTTTC    600

(1-388)R71A      505 GATATTTCTATTATCGAAATCGACGAAGTTGACGGCGAAAAAACCTTCGA    554
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           601 GATATTTCTATTATCGAAATCGACGAAGTTGACGGCGAAAAAACCTTCGA    650

(1-388)R71A      555 AGTTCTGGCAACCAACGGTGATACCCACCTGGGGGGTGAAGACTTCGACA    604
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           651 AGTTCTGGCAACCAACGGTGATACCCACCTGGGGGGTGAAGACTTCGACA    700

(1-388)R71A      605 GCCGTCTGATCAACTATCTGGTTGAAGAATTCAAGAAAGATCAGGGCATT    654
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           701 GCCGTCTGATCAACTATCTGGTTGAAGAATTCAAGAAAGATCAGGGCATT    750
```

DnaK(1-388) T225A

```
(1-388)T225A       1 ----------------------------------------AAACCTTCGA     10
                                                              ||||||||||
wtDnaK           601 GATATTTCTATTATCGAAATCGACGAAGTTGACGGCGAAAAAACCTTCGA    650

(1-388)T225A      11 AGTTCTGGCAACCAACGGTGATGCCCACCTGGGGGGTGAAGACTTCGACA     60
                     ||||||||||||||||||||||.||||||||||||||||||||||||||||
wtDnaK           651 AGTTCTGGCAACCAACGGTGATACCCACCTGGGGGGTGAAGACTTCGACA    700

(1-388)T225A      61 GCCGTCTGATCAACTATCTGGTTGAAGAATTCAAGAAAGATCAGGGCATT    110
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           701 GCCGTCTGATCAACTATCTGGTTGAAGAATTCAAGAAAGATCAGGGCATT    750

(1-388)T225A     111 GACCTGCGCAACGATCCGCTGGCAATGCAGCGCCTGAAAGAAGCGGCAGA    160
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           751 GACCTGCGCAACGATCCGCTGGCAATGCAGCGCCTGAAAGAAGCGGCAGA    800

(1-388)T225A     161 AAAAGCGAAAATCGAACTGTCTTCCGCTCAGCAGACCGACGTTAACCTGC    210
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           801 AAAAGCGAAAATCGAACTGTCTTCCGCTCAGCAGACCGACGTTAACCTGC    850

(1-388)T225A     211 CATACATCACTGCAGACGCGACCGGTCCGAAACACATGAACATCAAAGTG    260
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           851 CATACATCACTGCAGACGCGACCGGTCCGAAACACATGAACATCAAAGTG    900

(1-388)T225A     261 ACTCGTGCGAAACTGGAAAGCCTGGTTGAAGATCTGGTAAACCGTTCCAT    310
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           901 ACTCGTGCGAAACTGGAAAGCCTGGTTGAAGATCTGGTAAACCGTTCCAT    950

(1-388)T225A     311 TGAGCCGCTGAAAGTTGCACTGCAGGACGCTGGCCTGTCCGTATCTGATA    360
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           951 TGAGCCGCTGAAAGTTGCACTGCAGGACGCTGGCCTGTCCGTATCTGATA   1000

(1-388)T225A     361 TCGACGACGTTATCCTCGTTGGTGGTCAGACTCGTATGCCAATGGTTCAG    410
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK          1001 TCGACGACGTTATCCTCGTTGGTGGTCAGACTCGTATGCCAATGGTTCAG   1050


(1-388)T225A     411 AAGAAAGTTGCTGAGTTCTTTGGTAAAGAGCCGCGTAAAGACGTTAACCC    460
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK          1051 AAGAAAGTTGCTGAGTTCTTTGGTAAAGAGCCGCGTAAAGACGTTAACCC   1100
```

```
(1-388)T225A     461 GGACGAAGCTGTAGCAATCGGTGCTGCTGTTCAGGGTGGTGTTCTGACTG    510
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK          1101 GGACGAAGCTGTAGCAATCGGTGCTGCTGTTCAGGGTGGTGTTCTGACTG   1150
```

DnaK(1-388) H295D

```
(1-388)H295D       1 ----TTTCTATTATCGAAATCGACGAAGTTGACGGCGAAAAAACCTTCGA     46
                         ||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           601 GATATTTCTATTATCGAAATCGACGAAGTTGACGGCGAAAAAACCTTCGA    650

(1-388)H295D      47 AGTTCTGGCAACCAACGGTGATACCCACCTGGGGGGTGAAGACTTCGACA     96
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           651 AGTTCTGGCAACCAACGGTGATACCCACCTGGGGGGTGAAGACTTCGACA    700

(1-388)H295D      97 GCCGTCTGATCAACTATCTGGTTGAAGAATTCAAGAAAGATCAGGGCATT    146
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           701 GCCGTCTGATCAACTATCTGGTTGAAGAATTCAAGAAAGATCAGGGCATT    750

(1-388)H295D     147 GACCTGCGCAACGATCCGCTGGCAATGCAGCGCCTGAAAGAAGCGGCAGA    196
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           751 GACCTGCGCAACGATCCGCTGGCAATGCAGCGCCTGAAAGAAGCGGCAGA    800

(1-388)H295D     197 AAAAGCGAAAATCGAACTGTCTTCCGCTCAGCAGACCGACGTTAACCTGC    246
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           801 AAAAGCGAAAATCGAACTGTCTTCCGCTCAGCAGACCGACGTTAACCTGC    850

(1-388)H295D     247 CATACATCACTGCAGACGCGACCGGTCCGAAAGACATGAACATCAAAGTG    296
                     ||||||||||||||||||||||||||||||||.||||||||||||||||||
wtDnaK           851 CATACATCACTGCAGACGCGACCGGTCCGAAACACATGAACATCAAAGTG    900

(1-388)H295D     297 ACTCGTGCGAAACTGGAAAGCCTGGTTGAAGATCTGGTAAACCGTTCCAT    346
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           901 ACTCGTGCGAAACTGGAAAGCCTGGTTGAAGATCTGGTAAACCGTTCCAT    950

(1-388)H295D     347 TGAGCCGCTGAAAGTTGCACTGCAGGACGCTGGCCTGTCCGTATCTGATA    396
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK           951 TGAGCCGCTGAAAGTTGCACTGCAGGACGCTGGCCTGTCCGTATCTGATA   1000

(1-388)H295D     397 TCGACGACGTTATCCTCGTTGGTGGTCAGACTCGTATGCCAATGGTTCAG    446
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK          1001 TCGACGACGTTATCCTCGTTGGTGGTCAGACTCGTATGCCAATGGTTCAG   1050

(1-388)H295D     447 AAGAAAGTTGCTGAGTTCTTTGGTAAAGAGCCGCGTAAAGACGTTAACCC    496
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK          1051 AAGAAAGTTGCTGAGTTCTTTGGTAAAGAGCCGCGTAAAGACGTTAACCC   1100

(1-388)H295D     497 GGACGAAGCTGTAGCAATCGGTGCTGCTGTTCAGGGTGGTGTTCTGACTG    546
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
wtDnaK          1101 GGACGAAGCTGTAGCAATCGGTGCTGCTGTTCAGGGTGGTGTTCTGACTG   1150
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List of Publications and Patents

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