Role and mechanism of the Hsp70 molecular chaperone machines in bacterial pathogens

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Abstract

Heat shock proteins are highly conserved, stress-inducible, ubiquitous proteins that maintain homeostasis in both eukaryotes and prokaryotes. Hsp70 proteins belong to the heat shock protein family and enhance bacterial survival in hostile environments. Hsp70, known as DnaK in prokaryotes, supports numerous processes such as the assembly and disassembly of protein complexes, the refolding of misfolded and clustered proteins, membrane translocation and the regulation of regulatory proteins. The chaperone-based activity of Hsp70 depends on dynamic interactions between its two domains, known as the ATPase domain and the substrate-binding domain. It also depends on interactions between these domains and other co-chaperone molecules such as the Hsp40 protein family member DnaJ and nucleotide exchange factors. DnaJ is the primary chaperone that interacts with nascent polypeptide chains and functions to prevent their premature release from the ribosome and misfolding before it is targeted by DnaK. Adhesion of bacteria to host cells is mediated by both host and bacterial Hsp70. Following infection of the host, bacterial Hsp70 (DnaK) is in a position to initiate bacterial survival processes and trigger an immune response by the host. Any mutations in the dnaK gene have been shown to decrease the viability of bacteria inside the host. This review will give insights into the structure and mechanism of Hsp70 and its role in regulating the protein activity that contributes to pathogenesis.

INTRODUCTION

Heat shock proteins are the cellular response to stress at the molecular level. These proteins belong to a multi-gene family of proteins that differ in molecular size from 10 to 150 kDa and are found in all organisms from bacteria to humans. Heat shock protein families are of five types and are categorized according to their molecular size (i.e. Hsp100, Hsp90, Hsp70, Hsp60 and the small heat shock proteins). The two well-characterized families of these proteins are represented by molecular chaperones and proteases [1]. Members of the 70 kDa heat shock protein family (Hsp70) belong to a class of proteins termed molecular chaperones. Molecular chaperones constitute a group of proteins that can interact with varied types of protein substrates to assist in a broad range of folding processes. During stressful conditions, they have a significant role in helping to avoid the formation of wrong protein configurations which could lead to damaged molecules.

The two major chaperone systems in bacteria are the GroE and DnaK chaperones, and *Escherichia coli* is the major model micro-organism for studying these. The main heat shock protein is a chaperone of 70 kDa molecular mass known as Hsp70 or DnaK. It is present in the cytoplasm and plays a vital role by avoiding protein denaturation during the cellular stress response. Bacteria require DnaK for survival under stressful conditions, such as high temperature and challenge with heavy metals or antibiotics [2]. During these conditions, DnaK prevents the agglomeration of accumulated misfolded proteins and subsequently refolds them [3]. During infection, bacteria activate their heat shock genes to protect their cellular machinery from host defence mechanisms and thereby strengthen their virulence.

The GroE chaperone belonging to the Hsp40 family assists in the folding mechanism of individual protein molecules, whereas the DnaK chaperones act on native proteins, both unfolded and partially folded protein chains, by binding and controlling their configuration [4]. It also binds to hydrophobic regions of proteins that have become misfolded and aggregated due to cellular stress. Upon binding, the heat shock proteins either reverse the protein aggregation or promote the denaturation of the aggregate in order to avoid potentially toxic interactions between themselves and other proteins in the cell [2, 5].
The stress response is not the only function of Hsp70; it also has housekeeping functions in the cell and a significant role in maintaining normal growth and homeostasis. It is the most abundant cellular protein found under normal, non-stress growth conditions. Its prominent roles relate to the folding of newly synthesized polypeptides, the subcellular transport of proteins and vesicles, the formation and dissociation of protein complexes and the activation of proteolysis of misfolded proteins.

The major substrates of DnaK are:

1. Linear polypeptides that are either nascent or partially folded.
2. Exposed hydrophobic regions of completely or partially folded proteins.
3. Polypeptide strands composed of up to five consecutive hydrophobic residues.

Thus, Hsp70 broadly controls protein quality and yield during both normal and stress conditions [6].

**HIGHLY CONSERVED HSP70**

Hsp70 is one of the most evolutionarily conserved proteins and is found in all organisms from archaea to prokaryotic bacteria, plants and humans. The prokaryotic Hsp70 protein, the product of the bacterial dnaK gene, is approximately 50% identical to eukaryotic Hsp70 proteins in their amino acid sequence [6]. In *E. coli*, it is one of the most abundant cytoplasmic proteins and is approximately 1% of the total cellular protein content at conducive temperature. The first Hsp70 gene (dnaK) was identified in the laboratory of Costa Georgopoulos, when a mutation in one of the replication genes (AP) of *E. coli* was found because it became dormant when a mutation occurred in the *dnaK* locus [7].

The Hsp70 family is composed of multiple members with eukaryotes having numerous orthologues in a variety of cellular compartments. Higher eukaryotes express two homologues of the Hsp70 protein: constitutively expressed Hsc70 and stress-inducible forms (Hsp70). In humans, the Hsp70 multi-gene family includes the cytoplasmic and nuclear Hsc70 and Hsp70, respectively, endoplasmic reticulum-localized binding immunoglobulin protein (BiP or Grp78) and mitochondrial Mthsp75 or Grp75/mortalin. All are abundantly expressed during normal growth conditions [8].

**STRUCTURE AND SUBSTRATE BINDING OF THE HSP70**

The DnaK protein is made up of about 650 amino acid residues and has two functional domains; an N-terminal nucleotide-binding domain (NBD) of ~40 kDa and a C-terminal substrate-binding domain (SBD) of ~25 kDa, which are connected by a short linker [9] (Fig. 1). The NBD, also called the ATPase domain, is made up of two subdomains, I and II, which are further divided into regions a and b. The interaction of subdomains Ia and IIa with ATP molecules occurs through a nucleotide-binding fold that is related to those found in the proteins hexokinase, actin and glycerol kinase [10]. Amino acids present on both sides of the NBD form a cleft-like structure where ATP can bind. The hydrolysis of ATP to ADP drives conformational alterations in both domains. The SBD is also divided into two subdomains. The first is a β-sandwich subdomain of 15 kDa composed of two antiparallel β-sheets of four strands each. The second is a C-terminal α-helical subdomain composed of five helices, named A, B, C, D and E [11].

X-ray structures contain an extended conformation of substrate peptides bound between loops of the β-sandwich subdomain that incorporates a substrate binding pocket, which has high affinity towards neutral, hydrophobic amino acid residues and, with the α-helix sub-domain, acts as a ‘lid’ [12]. The Hsp70 cycle appears to revolve around cross-talk between NBD ATPase activity and the SBD substrate-binding activity, with Hsp70 binding tightly to the ATP [6]. An on–off switch is observed during substrate binding. When Hsp70 is in an ATP-bound state, the lid is open and the peptide is released rapidly. In contrast, when Hsp70 is in an ADP-bound state, the lid is closed and the peptide is released slowly due to tight binding by the SBD [13]. Therefore, hydrolysis of ATP determines the rate of reaction in the ATPase cycle. In particular, Hsp70 also requires specific monovalent (K+) and divalent (Mg2+) metal ions for ATP binding and hydrolysis. To stabilize these interactions and the spontaneous transition between the two ATP-bound states, Hsp70 requires the assistance of other proteins known as co-chaperones.

**HSP70 AND ITS CO-CHAPERONES**

Co-chaperones are a protein family that partake in the function of other chaperones by binding and delivering specific protein clients to these chaperones. They too exhibit chaperone activity because of their ability to prevent the aggregation of nascent polypeptides. Thus, they control the binding of Hsp70 to substrate polypeptides and provide stability to the complex [14]. These co-chaperones belong to the Hsp40 family and are expressed in both bacteria and eukaryotic cells.

The ATP-bound state has low binding affinity to the Hsp70 binding domain because SBD has an ‘open’ conformation, which may result in premature release of substrate...
peptide before it is folded into its proper conformation. The intrinsically weak ATPase activity of Hsp70 proteins necessitates collaboration with, and control by, co-chaperones of the J-domain protein family; known as J proteins, named after the *E. coli* DnaJ family of molecular chaperones. The J-domain proteins belong to a heterogeneous class of multi-domain proteins that contain an α-helical 70 amino acid consensus sequence located at the N-terminus of *E. coli* DnaJ [15]. They play a critical role in enhancing the ATPase activity of Hsp70 [16], by preventing the aggregation of non-native proteins and assisting in their delivery to Hsp70.

Another co-chaperone, dimeric GrpE, is the co-chaperone for DnaK. It is a nucleotide exchange factor (NEF) that removes ADP from DnaK and thereby controls its ATPase activity and reaction cycle [17]. The *dnaK* gene, along with the *dnaJ* and *grpE* genes, are together in an operon and thus can be co-transcribed to repair heat-induced damage of proteins [18].

In addition to the DnaK–DnaJ–GrpE system in *E. coli*, two newly discovered proteins that are also part of the Hsp70 type chaperone system are Hsc66, a stress 70 protein (second identified Hsp70 protein in *E. coli*), and Hsc20, a J-type accessory protein. However, a study showed that these proteins are part of a distinct molecular chaperone system because their cellular functions are totally separate from that of the DnaK–DnaJ–GrpE system [19].

THE CHAPERONE CYCLE

Hsp70 activity is characterized by multiple cycles of two coordinated activities that are carried out by the two domains of the protein: SBD and ATP binding (Fig. 2) [20, 21]. From these reports, it has been suggested that DnaJ first binds to a native polypeptide or unfolded protein in order to prevent its aggregation [5]. This non-aggregated or newly synthesized substrate is then delivered to DnaK [22]. This affiliation between DnaK and the polypeptide is stabilized by the J-domain; this domain catalyses the hydrolysis of DnaK–ATP to the DnaK–ADP complex, the latter of which has a higher affinity for unfolded polypeptides. The nucleotide exchange of ADP to ATP is then promoted by the binding of dimeric GrpE to DnaK, which weakens and ultimately terminates the DnaK–polypeptide interaction. This sequence of events has become a hypothesis for how Hsp70, DnaJ and the NEF can combine to help proteins fold into their active conformation [23].

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**Fig. 2.** Regulated ATPase cycle of DnaK and its coupling to substrate binding. In the first step of the ATPase cycle of DnaK, Hsp40 (1) binds to the substrate; the native protein (2) is then delivered to DnaK. The interaction between DnaK and polypeptide is stabilized by the J-domain (3) catalysing the formation of the DnaK–(ADP) complex. The DnaK–(ADP) complex becomes more receptive to unfolded polypeptides than the DnaK–ATP complex (4). The NEF removes ADP from Hsp70 and results in complex dissociation (5).
In eukaryotes, the constitutively expressed Hsc70 combines with Hsp40 (DnaJ homologue) and the NEF Bag-1 (GrpE homologue). Eukaryotic cells also have additional regulatory components such as Hip and Hop (60 kDa proteins), which act as adaptors between the Hsp70 and Hsp90 chaperone machineries. The function of Bag-1 is analogous to GrpE in bacterial cells [24].

**REGULATION OF HSP70**

In non-stressed cells, the concentration of heat shock proteins is quite low, whereas in stressed cells it tends to accumulate at high levels. The primary role of the Hsp70 protein under non-stressed conditions is to bind and modulate the conformational state and thereby activate certain native proteins that are involved in different regulatory processes. Under normal growth conditions, the concentration of DnaK in mesophilic *E. coli* (best growth at 37°C) is approximately equivalent to that of ribosomes (i.e. ~50 µM) so that it can interact with newly synthesized polypeptides [25].

The level of the heat shock response is directed by the interaction of the chaperone machinery, or DnaK–DnaJ–GrpE complex, with the heat shock sigma factor (σ32) subunit of RNA polymerase (encoded by the rpoH gene). σ32 is a transcriptional activator that recognizes the heat shock promoter at heat shock-related genes [26, 27]. It thereby helps unfolded proteins reach their functional three-dimensional configuration without actually interacting with the folded protein. σ32 has a very short half-life since it is degraded by proteases encoded by the hflB (ftsH) gene. Stress causes the σ32 subunit to be released from the DnaK–DnaJ–GrpE–σ32 complex, which triggers expression of heat shock genes [28]. When conditions are favourable, there is increased binding of DnaK to the heat shock transcription factor, σ32 of the RNA polymerase holoenzyme, facilitating the degradation of σ32 by the FtsH protease [29].

**ROLE OF HSP70 IN PATHOGENESIS**

When challenged by unfavourable temperatures, pH, antibiotics, free radicals or heavy metals in the host, prokaryotic DnaK is activated to increase the survivability of the bacteria. Pathogens take advantage of the host's heat shock proteins for their own benefit. It has been noted by Henderson et al. [30] that bacteria express many molecular chaperones on their cell surface and can release these into the extracellular environment, where they act as virulence signals (Fig. 3). Thus, microbial Hsp70 can be implicated in microbial pathogenesis, immune responses and cell death responses or apoptosis. These implications are found to be consistent with many studies, as discussed below.

**INVASION AND CELL ADHESION**

Hsp70 is bound to both host and microbial cell surface membranes and therefore facilitates easy attachment and colonization of pathogens. The interaction between bacteria and host epithelial cells is the first step in any bacterial infection. Upon entering the host, food-borne pathogens have to cope with stress factors found in the host gastrointestinal tract, including low pH, high temperature, bile salts, osmotic factors, etc. These interactions affect the expression of stress proteins in bacteria.

Mutations in DnaK make *Staphylococcus aureus* less infective and inhibit biofilm formation by *Streptococcus mutans* [31]. *Campylobacter jejuni*, a Gram-negative pathogen, usually survives in the gastrointestinal tract of birds, such as chickens, at 42°C. The mutant bacteria resulting from the inactivation of DnaJ lost *in vivo* culture viability and were unable to colonize chickens [32]. It has been reported that *Helicobacter pylori* can utilize cell surface Hsp70 as adhesions and the host molecules act as the ‘receptors’ for these ligands [33]. *H. pylori* has been reported to bind ligand sulfatide, and enteropathogenic *E. coli* Hsp70 binds to a ligand 3'-Sulfogalactosylsarcosylmide, in the host. Knockout of the dnaK–dnaJ operon in *Salmonella enterica* serovar Typhimurium creates mutants that show reduced culture viability. Mutant bacteria lacking dnaK and dnaJ did not survive in cultured epithelial cells and were not even able to colonize the intestines of mice [34]. However, impaired pathogenesis could be restored with the introduction of a functional copy of the *dnaK–dnaJ* operon, suggesting that Hsp70 is a significant factor in pathogenesis. High expression levels of both GroEL and DnaK have shown that they have protective roles in *E. coli* growth between 20 and 40°C [35]. In *E. coli*, the Hsp70 homologue stabilizes newly synthesized polypeptides and supports the assemblage of proteins into multiprotein complexes as well as their disassembly. However, improved performance in the host, i.e. better colonization and activity, was noted in probiotic bacteria or lactic acid bacteria only when they could express a molecular chaperone like Hsp70 and their other co-chaperones [36].
INTRACELLULAR SURVIVAL IN THE HOST

After crossing the epithelial barrier of the host, bacteria encounter immunological defensive molecules such as macrophages, which are considered to be the most stressful environments for the pathogens, including properties of phagosomes such as acidification, oxidative burst, etc. Successful pathogens adopt various strategies to resist the micobacterial activity of macrophages. The stress itself acts as a stimulus in inducing the expression of heat shock proteins [37]. Tissue-specific high expression of Hsp70 also helps the bacteria to proliferate within the macrophages [30]. It is reported that bacterial Hsp70 is required for the pathogenesis of *Mycobacterium* spp., which infects and replicates in host macrophages [32]. Under oxidative stress, the regulated expression of DnaK also helps the intracellular pathogen *Brucella* withstand the respiratory burst of phagocytes [38].

IMMUNE SYSTEM EVASION

When a pathogen attacks a host, its immune system is presented with an antigen that is not the single dominant one. Rather, due to the shared homology of antigen protein with the host (Hsp70 homologues Hsc70) molecules, the immune molecules were read either as antigen or autoantigens. This homology incorrectly directs the immune response against its own cells, which adds to further pathogenesis [39]. Reactive T cells and auto-antibodies against these heat shock proteins due to this molecular mimicry have been implicated in many autoimmune diseases such as inflammatory bowel disease [40]. In some intracellular pathogens, e.g. *Mycobacterium, Borrelia, Chlamydia* and *Legionella* spp., GroEL and DnaK have been well documented as likely antigens of infection and autoimmune disease [41]. A study by Retzlaff *et al.* [42] revealed that bacterial Hsp60 and Hsp70 can adjust to the immunity of the host by directly inducing the expression of cytokine genes in macrophages.

However, inactivation of *hspR* (heat shock protein receptor) and over-expression of DnaK by bacteria caused enhanced clearance of *Mycobacterium tuberculosis* in a murine model of tuberculosis. This can be attributed to the early signalling of the immune system with over-expression of DnaK, which possibly resulted in increased bacterial clearance. Hence, over-expression of heat shock proteins is not necessarily a pathway to virulence [43]. Bleotu *et al.* [44] proposed that Hsp70 antibodies could be used as diagnostic biomarkers for the intensity of generalized bacterial infections.

RESISTANCE TO ANTIBIOTICS

DnaK plays a key role in the pathogenicity of multidrug-resistant bacteria such as *Acinetobacter baumannii*, an opportunistic human pathogen. The DnaK machinery is involved in the antibiotic-resistant mechanism of *A. baumannii* under stress conditions [45]. *S. aureus* DnaK mutants showed reduced viability and increased susceptibility under stressed conditions in a host during systemic infection [46]. Strains of *S. aureus* with DnaK mutations showed increased sensitivity to oxacillin and metillin despite the fact that they are normally resistant to these antibiotics. In the same way, DnaK or DnaJ mutations in *E. coli* resulted in increased susceptibility to fluoroquinolones. Exposure to these antibiotics normally causes protein misfolding and aggregation. The Hsp70 protein sequesters these aggregates and assists in the refolding of these misfolded proteins [47]. It was also shown that antibiotic treatment usually eradicates commensal bacteria that have decreased Hsp70 expression.

ROLE OF HSP70 IN APOPTOSIS

The stress response and apoptosis are linked in the same way that disease pathologies and Hsp70 activity are linked. Disproportionate Hsp70 activity, either too high or too low, has been implicated in various diseases [48]. The over-expression of Hsp70 in tumours can lead to increased resistance against an apoptosis-inducing agent such as TNF-α. Over-production of Hsp70 also protects cancerous cells from various apoptotic and necrotic stimuli and hence confers a survival advantage. This theory can be related to many pathological processes [2, 49]. If Hsp70 levels are down-regulated in tumour cells, cell apoptosis or death can be induced.

HSP70 AS A MOONLIGHTING PROTEIN

From the above reports, it has been clarified that Hsp70 protein is a common moonlighting protein that assists which aid in bacterial virulence. Proteins or peptides having more than one biological activity are known as moonlighting proteins [50]. The major pathogenic species demonstrated to have the ability to modulate signals to human cells are now recognized [51]. DnaK functions as a signalling molecule in *M. tuberculosis* (Table 1).

In addition, in many other bacteria the Hsp70 protein acts as a cell surface receptor for various molecules. In *Neisseria meningitidis* [52] and *Bifidobacterium animalis* [53], the Hsp70 protein binds to human plasminogen. In *Lactococcus*

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<td>Signalled to leukocytes and CD8 lymphocytes, causing the release of the CC chemokines CCL3-5</td>
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<td>Hsp70 on myeloid cells stimulates monocytes to secrete CCL3-5 through a mechanism involving the transmembrane receptor of the TNF-α gene superfamily CD40</td>
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<td>Hsp70 also bound to the HIV co-receptor CCR5</td>
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lactis, DnaK binds to invertase of the host cell. These surface protein interactions lead to virulence of the pathogen. Moonlighting proteins increase virulence many times, by their involvement in complex interactions with the host.

CONCLUSION

The stress response is crucial for bacteria in adapting to changes in their physiological state. Hsp70 proteins, along with their co-chaperones and cooperating chaperones, constitute a complex network of protein-folding machines in prokaryotes and eukaryotic cellular compartments, and are expressed under both normal and stress conditions. This role of Hsp70 proteins is based on their ability to associate with unfolded substrate polypeptides to increase their stability in an ATP-controlled fashion.

Bacterial pathogens are one step ahead in exploiting heat shock protein Hsp70 to overcome tough challenges in the host cell milieu. Likewise, molecular chaperones control many approaches made by pathogens, from gaining entry into the host, to multiplying and surviving within the host. A recent approach to neutralizing bacterial infections is to exploit Hsp70 and its co-chaperones as potential drug targets that would sensitize prokaryotes to stress from antibiotics or host responses. The domains of DnaK are necessary for determining the correct folding of many polypeptides. Rational drug design can be utilized as a selective strategy to exploit Hsp70 and its co-chaperones as potential drug targets.

REFERENCES


