„Characterization of the molecular chaperone ClpB (Hsp 100) from the pathogenic bacterium Leptospira interrogans, the etiologic agent of leptospirosis"

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Bacterial molecular chaperone ClpB, belonging to the Hsp100 family, is an AAA+ ATPase (ATPase associated with a variety of cellular activities) which collaborates with the DnaK/DnaJ/GrpE (KJE) chaperone system by forming a bi-chaperone network. This bi-chaperone network solubilizes and reactivates aggregated proteins formed during stressful conditions.

The basic active unit of ClpB is a hexameric ring-shaped structure with a central channel. The disaggregation process involves translocation of polypeptide chains through this central pore. Each ClpB monomer is composed of an N-terminal domain (ND), responsible for substrate binding; two ATP binding domains (NBD-1, NBD-2) and an inserted coiled-coil middle domain (MD), crucial for interactions with DnaK. The NBD domains contain highly conserved motifs characteristic for AAA+ ATPases such as the Walker A (GX4GKT/S), Walker B (hy4DE), sensor 1 (N/T), sensor 2 (GAR) and the Arg fingers, coordinating ATP binding and hydrolysis.

Over the last decade, many researchers investigate a correlation between bacterial pathogen virulence and ClpB function. ClpB has been shown to be required for virulence of many pathogens, such as *Mycoplasma pneumoniae*, *Ehrlichia chaffeensis*, *Listeria monocytogenes*, *Frascinella tularensis* and *Leptospira interrogans* [**1**]. The Δ*clpB* mutants of *L. monocytogenes* and *L. interrogans* have been shown to be avirulent in experimentally infected laboratory animals. Moreover, the loss of functional ClpB in *L. interrogans* cells has resulted in bacterial growth defects under heat and oxidative stresses. It has been also demonstrated that ClpB proteins from *F. tularensis* and *M. pneumoniae* exhibit immunological properties. Furthermore, *clpB* expression is up-regulated in *E. chaffeensis* during infection of macrophage cell line. These observations revealed that there may be another chaperone-linked negative effect of ClpB. Namely, this protein has not only been produced during infection of the host organism but also it may be involved in this process [**1**]. It is presumed that inability of Δ*clpB* pathogen mutants to grow and induce disease symptoms may be caused by a reduced level of ClpB substrate proteins, which in turn may play an important role in the pathogen's survival. Therefore, the ClpB chaperone would allow pathogenic bacteria to adapt to conditions inside the host cells and achieving subsequent phases of infection, allowing their survival as well as the emergence of the disease.

The PhD thesis presented here, refers to this new aspect of ClpB chaperone function and is focused on investigation of the biological function of ClpB from *Leptospira interrogans* ($ClpB_{Li}$) pathogenic bacterium, the etiologic agent of leptospirosis in both humans and animals. Leptospirosis is one of the most widespread zoonoses in the world. More than 1 million cases of this disease occur worldwide each year, with mortality rate reaching even 20%. A high mortality rate is characteristic for tropical climate and flood areas, which is correlated with poorly developed health service. Furthermore, leptospirosis morbidity is higher in a hot climate, because leptospires are able to survive outside their host. *Leptospira* spp. reside in the proximal renal tubules of the kidneys of their carries and they are excreted with urine, and then contaminate soil and surface waters. The carriers may be rodents or small marsupials, but also domestic animals like cattle, pigs, horses, sheep, dogs. Leptospiral infection occurs by direct contact with urine or indirectly through an abraded skin or mucous membrane. However, human-to-human transmission happens relatively rarely. To the higher risk group of leptospirosis morbidity belong people who work with animals i.e. farmers, breeders, foresters, vets. Leptospirosis ranges from mild, flu-like symptoms in the first phase, to a serve (known as the Weil's disease) phase with renal and hepatic failure, pulmonary distress and finally yields to the death of the patient. It is worth to mention that this disease is currently also a serious economic problem in the agricultural sector, because of reduced milk production, frequent abortions, developmental defects and death in domestic animals.

The results of a French group published in 2011 (mentioned above)[1], that evidenced the involvement of ClpB in virulence of *L. interrogans*, had a key significance for the conceptualization of research problem in the presented PhD thesis. Then, it was not attempted to describe properties of $ClpB_{Li}$ which might relate its specific role during *Leptospira*-infections with its molecular chaperone function and involvement in disaggregation and reactivation of aggregated proteins. Therefore, the main aim of this PhD thesis was to characterize a chosen set of $ClpB_{Li}$ properties.

In the beginning, the amino-acid sequence alignment of $ClpB_{Li}$ and well-characterized ClpB from *Escherichia coli* ($ClpB_{Ec}$) was performed by using the Clustal software. The $clpB_{Li}$ gene encodes a protein of 860 amino acid residues with a molecular mass of 96.33 kDa. Based on bioinformatic analyses, it was revealed that $ClpB_{Li}$ protein shows a multi-domain

[1]K. Lourdault, G.M. Cerqueira, E.A. Wunder Jr., and M. Picardeau (2011) Inactivation of *clpB* in the pathogen *Leptospira interrogans* reduces virulence and resistance to stress conditions. Infect. Immun. 79: 3711-3717.

organization similar to that of $ClpB_{Ec}$, and contains an N-terminal domain ($ND1\text{-}_{145aa}$), two nucleotide binding domains ($NBD\text{-}1_{161\text{-}342aa}$, $NBD\text{-}2_{560\text{-}768aa}$) and middle coiled-coil domain ($MD_{393\text{-}527aa}$) [**2**]. The total sequence identity between $ClpB_{Li}$ and $ClpB_{Ec}$ is only 52% (27.7% within ND; 45.3% within MD; 72% within NBD-1 and 65.7% within NBD-2). Therefore, the most highly conserved are the NBD domains [**1**].

Next, a construct expressing *clpB*$_{Li}$ and providing efficient overproduction of recombinant $ClpB_{Li}$ protein in *E. coli* cells was obtained by using a pET28b+ expression plasmid [**2**]. The protein was purified using two-step procedure: immobilized metal affinity chromatography (with Ni-NTA agarose) and gel filtration (Superdex 200). The final purified product was validated with an LC-MS-MS/MS (Liquid Chromatography-tandem Mass Spectrometry) analysis [**2**] and used in subsequent studies [**2, 3**]. First, the immune reactivity of $ClpB_{Li}$ with serologically positive sera from cattle and rabbits experimentally infected with chosen *Leptospira* spp. strains was tested by Western blotting and an ELISA assay (Enzyme-Linked Immunosorbent Assay) and compared to the sera from uninfected healthy controls. The results suggest that a *Leptospira* infection induces the humoral immune response of the host, because of an increased level of specific anti-$ClpB_{Li}$ antibodies in the sera collected from infected animals. Additionally, $ClpB_{Li}$ was detected in the infected hamster kidney tissue using specific anti-$ClpB_{Li158\text{-}334}$. This result indicates that $ClpB_{Li}$ is produced during an experimental infection of the host and points to $ClpB_{Li}$ involvement in the virulence of *L. interrogans* [**2**].

In the next step, the secondary structure of the $ClpB_{Li}$ protein and its thermal stability was estimated by performing CD spectroscopy (Circular Dichroism) [**3**]. The obtained data show the $ClpB_{Li}$ is folded into a structure that is dominated by α-helices which is in agreement with the secondary structure of $ClpB_{Ec}$. Moreover, based on the collected CD spectra, the melting temperature (Tm) was defined as 67 °C. The high Tm value shows that $ClpB_{Li}$ is thermodynamically stable.

Subsequently, an ability to self-association of $ClpB_{Li}$ in the presence of nucleotides: ADP, ATP analogues like ATPγS, AMP-PNP or in their absence and also in a buffer with low ionic strength was tested [**3**]. By analytical ultracentrifugation and sedimentation velocity measurement, it was shown that full oligomerization of $ClpB_{Li}$ occurs in the presence of a non-hydrolyzable ATP analogue i.e. ATPγS, and then $ClpB_{Li}$ forms hexamers. ADP also induces $ClpB_{Li}$ self-assembly, but does not yield to formation of hexamers. Monomers appear mostly in the absence of a nucleotide or in the presence of another non-hydrolyzable ATP analogue, AMP-PNP, and in the low ionic strength buffer. The effect of the mentioned above

nucleotides on the $ClpB_{Li}$ structure was also investigated by monitoring changes in its proteolytic degradation using trypsin [**3**]. The $ClpB_{Ec}$ protein served as a control in this experiment. It was observed that the presence of ATP, ATPγS, AMP-PNP and ADP, afforded a varying degree of protection of ClpB proteins against trypsin activity, while digestion of both chaperones without any nucleotide present had progressed in time. Based on these observations, ClpB's undergo structural changes upon nucleotide binding. However, it should be emphasized that $ClpB_{Li}$ in the presence of either ATPγS or ADP was more resistant to proteolysis than $ClpB_{Ec}$. This means that ATPγS and ADP stabilize the $ClpB_{Li}$ oligomers more effectively. Overall, the extent of the $ClpB_{Li}$ protection against trypsin in the presence of different nucleotides correlates with the capability of this protein to form oligomers. The hexamerization of ClpB in the presence of a nucleotide is crucial for ClpB's biological function and for its involvement in disaggregation and reactivation of aggregated proteins [**3**].

The next aim was to a determine the ATPase activity of $ClpB_{Li}$ and investigate the influence on this activity of unstructured polypeptides (κ-casein, polylysine) and aggregated glucose-6-phosphate dehydrogenase (G6PDH) [**3**]. It was found that $ClpB_{Li}$ exhibits a slightly lower basal ATPase activity than $ClpB_{Ec}$. Moreover, $ClpB_{Li}$ ATPase activity is stimulated by κ-casein and polilysine, but the presence of aggregated substrate, G6PDH, did not significantly affect the ATPases of $ClpB_{Li}$ and $ClpB_{Ec}$ [**3**].

Next, the $ClpB_{Li}$ disaggregating activity in the presence or absence of the *E. coli* DnaK/DnaJ/GrpE chaperone system was tested *in vitro* [**3**]. For this purpose, a known model substrate of $ClpB_{Ec}$ i.e. chemically and thermally denaturated G6PDH and two new substrates: thermally aggregated FBP aldolase (Fda) and inclusion bodies of VP1-β-galactosidase isolated from *E. coli* cells were used. Based on the obtained result it was concluded that $ClpB_{Li}$ is more effective in reactivating aggregated proteins without the KJE system when compared to $ClpB_{Ec}$ and the presence of KJE proteins did not significantly affect the disaggregase activity of $ClpB_{Li}$, like in the case of $ClpB_{Ec}$. These observations have shown that $ClpB_{Li}$ may mediate protein disaggregation of some substrates independently from the KJE chaperone system [**3**].

Furthermore, the disaggregase activity of $ClpB_{Li}$ was investigated in *E. coli* MC4100 Δc*lpB* cells [**3**]. From the literature, it is evident that the lack of a functional $ClpB_{Ec}$ decreases the growth rate of bacteria at 45 °C and inhibits cell survival after exposure to a severe heat shock (50 °C). Therefore, it was checked whether $ClpB_{Li}$ can functionally substitute for $ClpB_{Ec}$ by performing a complementation assay. To achieve this goal, the *clpB*$_{Li}$ gene was cloned into a low-copy pGB2 plasmid, and the resulting plasmid was introduced into the *E.*

coli MC4100 Δ*clpB* mutant and then its growth (at 45 °C) and survival (at 50 °C) were analyzed. It was observed that $ClpB_{Li}$ protein did not rescue the *E. coli* Δ*clpB* mutant under heat shock conditions because (1) bacteria producing $ClpB_{Li}$ protein had a decreased growth rate at 45 °C; (2) $ClpB_{Li}$ did not enable growth of *E. coli* MC4100 Δ*clpB* bacteria during severe heat shock. These results indicate that $ClpB_{Li}$ is not able to functionally substitute for $ClpB_{Ec}$, probably because of lack of cooperation with *E. coli* KJE chaperone system due to the loss of interactions between $ClpB_{Li}$ MD domain and DnaK from *E. coli* [**3**]. This result emphasizes species-specificity of molecular chaperones.

The next research task was to investigate the nucleotide's influence (ATP and ATPγS) on interactions of $ClpB_{Li}$ with the aggregated substrate, G6PDH [**3**]. It is known that ATPγS binds to NBD domains of ClpB, but it is not hydrolyzed, which induces a "frozen" ClpB state. The obtained results indicated that the presence of a nucleotide changes $ClpB_{Li}$ affinity to the aggregated substrate. $ClpB_{Li}$ more effectively interacts with the substrate in the presence of ATPγS. On the other hand, in the presence of ATP the $ClpB_{Li}$-substrate state is more dynamic because of ATP hydrolysis and G6PDH reactivation. Under these conditions $ClpB_{Li}$ exhibits lesser capability for stable aggregate binding [**3**].

This PhD thesis also includes a preliminary identification of putative physiological $ClpB_{Li}$ protein substrates isolated from *Leptospira* lysates treated under stressful conditions (37 °C, 4 h; 42 °C, 2 h). For this purpose, a plasmid encoding a $clpB_{Li}$ gene with two point mutations within NBD-1 and NBD-2 domains was constructed [**4**]. It is known from the literature, that replacement of the conserved glutamic acid in each of the two Walker B motifs in NBD-1 and NBD-2 with alanine results in ATP binding, but not its hydrolysis. This ClpB variant acts as a "substrate trap" in the presence of ATP, because it permanently binds proteins interacting with ClpB. Using purified $ClpB_{Li}$-Trap (E281A/E683A) protein, cell lysates from *L. interrogans* and pull-down strategy coupled with LC-MS-MS/MS and bioinformatics analyses, 68 proteins were identified which form stable complexes with $ClpB_{Li}$. Most of the described proteins play a key role in major metabolic pathways of the bacterial cell, such as the glycolysis-gluconeogenesis, tricarboxylic acid cycle, or amino acid biosynthesis and fatty acid metabolism. Based on these results, it has been proposed that substrates for $ClpB_{Li}$ are mainly key metabolic enzymes. Furthermore, some of the identified proteins are involved in other important cellular processes such as ribosome biogenesis, translation, transcription or chemotaxis [**4**]. It is highly probable that the identified enzymes may have an important impact on growth and pathogenicity of *Leptospira*. Therefore, it has

been proposed that the role of $ClpB_{Li}$ in virulence of *Leptospira* is to protect the activity of metabolic enzymes and control *Leptospira* cell metabolism during stressful conditions.

In summary, in this PhD thesis, for the first time the detailed characterization of $ClpB_{Li}$'s properties, a protein from pathogenic spirochaete – bacterium with still not well known biology was carried-out. The obtained results suggest, that ClpB is synthesized during infection and induces humoral immune response of the host system. Moreover, it was shown, that $ClpB_{Li}$ forms hexameric assemblies that are stabilized in the presence of a nucleotide and may interact with protein aggregates in the ATP-bound state. A valuable finding is that $ClpB_{Li}$ mediates protein disaggregation independently from the KJE system, which may be necessary for rapid adaptation to intracellular life conditions during the infectious process. The structural and biochemical characterization of $ClpB_{Li}$ and proteomic analyses provided new information, which may explain this protein's role in the leptospiral virulence and pathogenesis of leptospirosis. In addition, these data open new opportunities for developing novel, more efficient antibacterial therapies in the future.

References:

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