

HtrA from *E. coli* is a model protein of the HtrA family of proteases. Bacterial HtrA's protect cells from harmful stressful agents and are important for virulence of many pathogenic strains. Therefore they are considered as a potential target for antimicrobial treatment. However, designing of a specific inhibitor molecule requires detailed knowledge concerning structure and mechanism of regulation studies of the target virulence factor.

The structure of the HtrA protease has been relatively well characterized and crystal structures of this protein are available. However, this enzyme has a very complicated mechanism of regulation, still not fully understood. HtrA can be activated by means of allosteric activation and/or temperature shift. At the physiological conditions, HtrA exists as a proteolytically inactive hexamer. It is believed that the regulatory loops LA play an important role in maintaining this inactive structure.

Binding of appropriate substrates activates the protease. It leads to transmission of an allosteric signal, resulting in reorganization of the structure of the whole enzyme. In consequence the catalytically active molecule is formed. The regulatory loops (L1, L2, L3, and LD) are involved in this process. Due to the high flexibility of LA loop, its part containing residues from 55 to 79 has not been traced in the crystal structure of HtrA. Hence, precise interactions in which the LA loop is involved are not known. The particular residues of the LD loop that are responsible for allosteric signal transition also remain unknown.

The aim of this work was to determine the mechanism of action of two regulatory loops playing opposite functions: inhibitory LA loop and activation LD loop. For this purpose, the point mutations, potentially affecting the inactive (LA loop) or active form of the enzyme (LD), were introduced to HtrA. The HtrA variants with undisturbed secondary structure, determined by the means of far-UV circular dichroism, were analyzed.

Due to a lack of information about the structure of LA, a theoretical structural model of this LA loop has been obtained in cooperation with dr. A. Giędoń from the Faculty of Chemistry of UG (htrA\_unress, supplementary information in Figaj et al., 2014). My role was to (1) provide experimental data concerning the proteolytic activity of mutated HtrA proteins to improve this model and (2) verify the correctness of the model using site-directed cross-linking (Cys–Cys). The key interactions maintaining the inactive HtrA structure were identified during work on this model. It was shown that the LA loops interact with each other within the hexamer by means of hydrophobic residues. A particularly important role was assigned to residues F46, F49, F50, F63, F68, which together with P62 and P67 form hydrophobic cluster located in the central part of the hexamer. The hydrogen bond network formed by means of the amino acid residues from the LA, L1 and L2 loops, was also

characterized. The inhibitory interactions of the LA loop (the residues Q47 and F68) with the other regulatory loops were pointed out. Moreover, the important role of the N-terminal part of the LA loop (the region around residue 44) in the maintenance of the loop rigidity was demonstrated using tryptophan fluorescence quenching and size exclusion chromatography. The locally increased flexibility of the LA loop resulted in destabilization of the hexamer and facilitated formation of the higher order oligomeric structures, but also led to protein autodegradation.

In the second part of this work, the role of the LD loop in the activation process of HtrA was characterized. It was demonstrated that maintenance of the structure and stiffness of this loop was crucial for the proteolytic activity of HtrA. It was also shown, that residues F171 and L173 are responsible for interaction with the L2 loop from the opposing subunit. Furthermore, the LD loop is a part of the activation cluster – the hydrogen bond network stabilizing the active conformation of the protein. It was observed, that stabilization of this interactions network resulted in hyperactivity of the HtrA protein, associated with a shift of the allosteric equilibrium towards an active form of the enzyme regardless of the presence of a substrate. Expression of the HtrA variant, characterized by an enhanced and uncontrolled proteolytic activity had a negative impact on bacterial survival at physiological temperature 37°C.

Summing up, data obtained in this work contribute to deeper understanding of the roles of the regulatory LA and LD loops in the activation process of HtrA. Moreover, the results of my study were basis for the development of the theoretical model of the LA loop structure.