San Hadži, Structural and thermodynamic basis of transcription regulation of the *higBA2* toxin-antitoxin module from *Vibrio cholerae*

Toxin-antitoxin modules are small bacterial operons (genetic systems) which code for a toxin and an antitoxin protein. During poor growth conditions such as presence of antibiotics or nutrient depletion, the antitoxins are degraded and the toxins target one of the key cellular functions thus shifting the cell into a hibernating state. In this state, also known as the persister phenotype, the cells are dormant and tolerant to the antibiotic treatment. Upon improvement of growth conditions, the cells can (re)produce the antitoxin which binds the toxin into a nonactive toxin-antitoxin complex. Thereby the cells shift back to normal fast-growing state, which can, in case of pathogenic bacteria, lead to reoccurrence of the infection. Cells control the activity of toxin-antitoxin modules through different regulatory mechanisms.

The aim of this thesis is to elucidate the regulatory mechanism of the mostly unstudied *higBA2* toxin-antitoxin module from the human pathogen *V. cholerae.* I used x-ray diffraction techniques to obtain structural information on toxin, antitoxin and different complexes of the *higBA* module. Using biophysical methods I characterized interactions between these macromolecules in order to understand what drives conversion from one macromolecular complex into another. Synthesis of these two approaches is development of a mathematical model of the *higBA2* regulation.

There are three main results in this thesis. The first is that the *higBA2* module is regulated by a novel *anticooperative* regulatory mechanism, which I thoroughly characterize in Chapters 2 and 3. In addition to the regulation of toxin via antitoxin, there is another level of regulation which controls the protein concentrations in a way that there is more antitoxin than toxin. This is achieved by a mechanism that regulates transcription of the *higBA2* operon as a function of molar toxin/antitoxin ratio. The ratio-dependent regulation has been observed for several other toxin-antitoxin modules, however the *higBA2* module has a different, and much simpler mechanism. The anticooperative mechanism relays on strong transcription repression by the HigA2 antitoxin at low toxin/antitoxin ratios, while binding of toxin to the antitoxin is associated with a negative cooperativity leading to the de-repression at high toxin/antitoxin ratios. At the molecular level the intrinsically disordered domain of the HigA2 antitoxin plays a key role in establishing the regulatory mechanism.

The second main result is a comparison of all currently well-characterized toxin-antitoxin regulatory mechanisms (Chapter 4). For each of the regulatory mechanisms I calculated their phase space (all possible states) as a function of total toxin and antitoxin concentration. Using this method I investigated the main features of the toxin-antitoxin regulatory mechanisms and proposed their biological significance. By analyzing what drives the ratio-dependent regulation I propose using different names for what is now known simply as conditional cooperativity regulation. These are: conditional avidity regulation, conditional avidity regulation with cooperativity and conditional cooperativity regulation. These mechanisms are more complex compared to the anticooperative regulation and I discuss their possible benefits.

The third main result is development of a method for thermodynamic characterization of folding of the intrinsically disordered proteins (Chapter 5). It appears that the primary regulatory mechanism is high-affinity toxin-antitoxin interaction. The HigA2 antitoxin, like many other antitoxins, folds upon binding the toxin. The thermodynamics of such coupled process are not well understood because it is difficult to study each of the processes (folding or binding) separately. I used 2,2,2-trifluoroethanol (TFE) to fold the HigA2 intrinsically disordered domain and investigated the temperature-induced unfolding as a function of TFE concentration. These experiments were analyzed using the Lifson-Roig theory of helix-coil transition in order to obtain thermodynamic parameters for folding of the intrinsically disordered domain independent of toxin binding.