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Structural perspectives of natural and directed protein evolution

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Academic dissertation

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Abstract

Proteins are central agents for all cellular processes of life. They confer shape or enable motility, act as defence or a means of aggression, and catalyse a plethora of chemical reactions. A protein's function is typically determined by its three-dimensional structure and, indeed, many human diseases arise when proteins misfold or denature prematurely. Yet life has continuously evolved since its inception, implying that there are underlying principles governing which changes are viable and which are not. By determining the atomic structure of proteins, we can follow these evolutionary trajectories and unveil how they influence biological function. With cutting-edge scientific methods, such insights can, in turn, be used to guide the design of new drugs, or, due to recent advances in engineering, the synthetic design of entirely novel proteins.

In this thesis, I aim to contribute to the fundamental understanding of structural biology by exploring aspects of both natural and directed protein evolution. I used state-of-the-art cryo-Electron Microscopy (cryo-EM) to characterize protein structures, enabling detailed analyses of how evolution navigates structural constraints to repurpose protein folds and optimize molecular interactions.

In the first project of this thesis, I investigate the emergence of novel ribosomal proteins in Microsporidia. By analysing high-resolution structural models of their ribosomes, I observed a progressive reduction of ribosomal RNA, loss of expansion segments, and truncation of ribosomal proteins. To maintain ribosomal integrity, microsporidia acquired new proteins that compensate for the loss of stabilizing interactions. I discovered that these proteins were in essence 'evolved fold-switchers': homologs that adopted highly dissimilar structures, with inverted termini, despite occupying the same ribosomal niche. Thus, these findings represent a rare example where a proteins' function and cellular environment are more conserved than its fold.

The second project shifts the focus towards directed evolution. Here, an affinity protein, CaRA_{EGFR}, was engineered to recognize the cancer marker epidermal growth factor receptor (EGFR) via a mechanism calibrated to high calcium concentrations typically found in the bloodstream. As a result, CaRA_{EGFR} bind its target with high affinity whilst in circulation but loses this affinity after being trafficked into cells via endocytosis. This conditionally regulated mechanism greatly improved CaRA_{EGFR}'s potential to act as a targeting unit for protein-drug conjugates, and we demonstrated potent cytotoxicity in EGFR-expressing cancer cells when CaRA_{EGFR} was coupled to a drug. Although the complex of CaRA_{EGFR} and EGFR corresponds to a size of only ~80 kDa, I could confirm their interaction using single-particle analysis (SPA) cryo-EM.

In the third project, I expand on these findings by providing a more detailed structural characterization of the CaRA_{EGFR}:EGFR complex using cryo-EM. I observed that CaRA_{EGFR} engaged with EGFR via a hydrophobic cleft in domain III and compared this mode of recognition to other known EGFR-binding proteins. Complementary NMR experiments were used to probe calcium-dependent structural changes, allowing us to propose an allosteric mechanism in which environmental calcium concentrations modulate this interaction.

The fourth project returns to natural protein evolution. Here, I investigated the biochemical properties of BmdE, a nuclease involved in interspecies biofilm dispersion. Using cryo-EM, I solved the structure of its nuclease core, revealing a novel, extended phosphodiesterase domain comprising an a/b sandwich motif. Notably, the cryo-EM data contained additional densities suggesting an intermediate reaction state 'trapped' after DNA cleavage. The structural information was then used to establish an evolutionary context, revealing that BmdE-like nucleases represent a kingdom-spanning protein family rather than a newly evolved, species-specific mechanism.

In summary, this thesis explores diverse structure-function relationships across evolutionary scales, ranging from the *de novo* birth and subsequent fold switching of ribosomal proteins to a rationally engineered, conditionally regulated affinity protein for cancer treatment. Collectively, this work contributes new insights into the adaptability of protein architectures that may guide future efforts in structure-based drug design, protein engineering and biofilm control.

Keywords: Structural Biology, Evolutionary adaptation, cryo-EM, Microsporidia, Biofilms, Protein-drug conjugates

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