ABSTRACT

Despite the growing interest in green chemistry and enzymatic catalysis, the widespread industrial application of biocatalysts remains limited. This is primarily due to the narrow spectrum of reactions they catalyze and their high sensitivity to factors such as pH and temperature. However, modern protein design and engineering methods allow for the creation of highly efficient catalysts dedicated to specific reactions, capable of operating under mild conditions.

In this context, miniproteins represent a particularly promising group of compounds. These are polypeptides with a molecular weight below 10 kDa, characterized by a well-defined and highly stable tertiary structure. They can be also designed entirely *de novo*, leading to development of topologies not found in nature. These features make them excellent scaffolds for introducing functional amino acid residues.

In this work, two strategies for designing catalytically active miniproteins capable of promoting the aldol reaction between 4-nitrobenzaldehyde and phenylacetaldehyde were investigated.

In the first stage of the research, catalytic residues — lysine and tyrosine — were introduced into the natural miniprotein **MvaT**. 15 different combinations of their arrangement were analyzed to select the most effective active site architecture. The best variant was then iteratively optimized, achieving a 1700-fold reaction acceleration.

In parallel, work was carried out on the *de novo* design of miniproteins, into whose helices a cyclic β-amino acid (*trans*-ACPC) was introduced, which rigidifies the structure and promotes its folding. The construction of completely new structures expanded the set of available scaffolds. The sequences were generated using Rosetta software. The peptides were synthesized on a solid support, and the structural analysis was based on circular dichroism and nano-differential scanning fluorimetry (nanoDSF) methods, with 2D NMR measurements performed for selected compounds.

As a result, two classes of highly stable miniproteins with distinct topologies were obtained: HHEEE (helix-helix-β-sheet) and HEEEH (helix-β-sheet-helix). Through rationally selected mutations of the most promising variants, miniproteins capable of a 3000-fold reaction acceleration were obtained, and the structural studies resulted in a partially solved structure of one of the inactive scaffolds.

The conducted research not only deepens the understanding of the relationship between structure and activity of miniproteins in reactions extending beyond model substrate sets, but

also represents another step toward developing an effective methodology for designing catalysts with exceptional properties.