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To the
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Review report of the dissertation

submitted by

Mgr. Kateřina Švehlová

entitled

"Development and characterization of light-producing deoxyribozymes "

for defence in the study program

Molecular and Cellular Biology, Genetics and Virology

I confirm that I do not have any bias or conflict of interest in relation to the author of the work.

Introduction

The research summarized in the dissertation thesis submitted by Ms Katerina Svehlova was carried out under supervision of Dr. Edward Curtis at the Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences in Prague. The thesis presents original research results in the area of nucleic acid chemistry and chemical biology, with a special focus on the in vitro selection and characterization of functional nucleic acid enzymes, in particular deoxyribozymes that have been evolved to catalyze a chemiluminescent reaction.

The work aimed at the development of deoxyribozymes that mimic light-producing enzymes for biosensor applications. The in vitro selection of a functional nucleic acid from a random library was designed to yield a phosphatase function to dephosphorylate the chemiluminescent substrate CDP-Star, in analogy to alkaline phosphatase enzymes, resulting in the emission of blue light. Ms Svehlova successfully identified several deoxyribozymes and optimized the lead candidate by re-selection and rational mutagenesis. The structural and functional properties of the new DNAzymes – called Supernova – were studied in detail, resulting in profound insights into sequence requirements and optimal reaction conditions, as well as the development of a deoxyribozyme variant for the detection of ligand binding events.

The results of the very successful thesis have recently been published in internationally highly recognized journals.

Ms Svehlova is the first author of a seminal paper reporting the first SuperNova DNAzyme in *Angewandte Chemie Int. Ed.*, and co-author of an additional publication in *ChemBioChem* describing the optimization of the light-producing deoxyribozyme. Moreover, she is co-author of four additional research articles published in prominent journals, including *Nucleic Acids Research* on the specificity of G-quadruplex motifs. The results reported in these publications deal with research questions that are not directly related and are not discussed in the thesis.

Research scope

The in vitro selection of functional DNAzymes from synthetic nucleic acid libraries presents a daunting but rewarding challenge that has immense potential for providing new biomolecular catalysts, fundamental insights into the scope of DNA-catalyzed reactions as well as diagnostic tools for future applications. Ms Svehlova has taken up the challenge and successfully developed a chemiluminescent deoxyribozyme. The selection design mimics previous in vitro selection strategies for self-phosphorylating kinase deoxyribozymes using nucleotide triphosphates as phosphate source. The key innovation here was to use a known alkaline phosphatase substrate, the 1,2-dioxetane CDP-Star, which upon enzymatic dephosphorylation spontaneously disintegrates with emission of light. The selection strategy was focused on capturing the phosphorylated DNA catalyst, a prerequisite for the separation of active DNAzyme candidates. Ms Svehlova successfully implemented the original in vitro selection and obtained the first candidate sequences by a traditional cloning and sequencing approach. Several DNAzymes were obtained that showed activity in the ligation assay, proving that they acquired a phosphate during the reaction, but the chemiluminescence enhancement response was marginal for all but one candidate. This promising DNA sequence was then randomized and used for reselection, which was then analyzed by the powerful method of Illumina sequencing (NGS). Careful comparative sequence analysis together with rational mutagenesis resulted in the highly active DNAzyme Supernova that showed more than 6000-fold rate enhancement. This deoxyribozyme was biochemically characterized with respect to its kinetic parameters and engineered into a chemiluminescent DNA sensor that was activated upon hybridization to the target oligonucleotide. These results of the original contributions by Ms Sveholva are clearly documented in chapter 4 of the thesis.

Methods, results and conclusions

The methods and techniques applied in the execution of the work are fully appropriate, and the described results are clearly supported by the data presented. Required control reactions have been included and the results are carefully interpreted, taking into account possible background reactions and non-specific activation. The proposed triple-helix is a novel and highly interesting structural motif of the DNA catalyst. Another interesting finding pertains to the required cofactor of the supernova deoxyribozyme, which was selected in the presence of lead, cerium and zinc as divalent metal ion cofactors, but later found to only require Zn^{2+}. The kinetic parameters of supernova were determined under various reaction conditions, resulting in optimized parameters for the DNA-catalyzed reaction. As described in this thesis, the supernova deoxyribozyme is limited to a single turnover, as it gets self-phosphorylated, and the phosphate is not released from the catalyst. It remains unclear if/how this aspect has been considered when discussing "Michaelis-Menten kinetics". It would be interesting to develop ideas how this limitation may be overcome in future editions of supernova.

Structure of the thesis, presentation and language

The thesis is clearly structured and written in excellent English language. The introduction and the presentation of the state of the art in chapter 2 demonstrate that Ms Svehlova has excellent knowledge of the relevant literature. She included a brief historical perspective on the field in general, summarized current knowledge on natural and artificial ribozymes and deoxyribozymes, and highlighted the required methods and methodological developments for in vitro selection, analysis and characterization, and included the appropriate references. The comparison of existing designs of fluorescent nucleic acid sensors led her to a succinct description of the aims of her thesis in section 3. The individual steps of the project design are clearly laid out. However, rationalization for the choice of the CDP-Star substrate and possible alternatives could have been elaborated in more detail. Chapter 4 contains all the results, and these are very well documented. While the results published in *Angewandte Chemie* on the discovery of the Supernova deoxyribozyme are narrated in subchapters 4.1-4.6, the optimization in chapter 4.7 is presented in the form of an accepted manuscript, meanwhile published in *ChemBioChem*. This mixture in presentation style within one chapter of the thesis seems a bit unusual to me, but it may well be in line with the formal requirements. The Figures Schemes and Tables throughout the thesis are clearly labeled, and the legends of Figures showing original data provide the necessary detailed information for the reader. Only in some cases some more explanation would have been helpful, for example in Figures 12 and 27d, where it remains unclear how "rate enhancements" are defined and calculated. In chapter 5, the results of the thesis are set into perspective and discussed in the context of future directions for applications and expected improvements of future generations of supernova deoxyribozymes. Section 6 contains a concise concluding paragraph, before the experimental details, materials and methods are clearly laid out in Chapter 7, followed by the list of references and the supporting information of the accepted manuscript as an appendix.

Evaluation summary

In summary, the thesis makes highly important and original contributions to the subject of nucleic acid catalysis and fully meets and exceeds the general requirements for the academic work of a PhD dissertation. For these reasons, I fully recommend without hesitation this dissertation thesis of Ms Katerina Svehlova for acceptance to the defence in the doctoral study program Molecular and Cellular Biology, Genetics and Virology at the Faculty of Science of Charles University Prague.

Würzburg, 08.06.2022

Prof. Dr. Claudia Höbartner