Opinion

on the dissertation work of Elitsa Hristova Boteva by Associate Professor Dr. Anastas Georgiev Gospodinov, Institute of Molecular Biology "Acad. R. Tsanev", BAS

The dissertation work of Elitsa Hristova Boteva, titled *"DNA deglycating activity of the glycolytic enzyme phosphoglucose isomerase"*, was done at the Institute of Molecular Biology "Academician Rumen Tsanev" at BAS, under the supervision of Professor Dr. Rumyana Mironova. The dissertation was submitted for the award of the educational and scientific degree "Doctor."

Elitsa Boteva's dissertation represents an investigation into the role of the enzyme phosphoglucose isomerase in the deglycation of DNA and its role in the protection of genomic stability. It contains 177 pages, including 3 tables, 55 figures, and an extensive literature review with 287 sources.

The presented research impresses with its depth and scope—from the identification of the enzyme responsible for DNA deglycation in *E. coli* and humans to the details of the reaction mechanism of this enzyme.

The literature review provides a comprehensive and systematic overview of the topic related to glycation of biological macromolecules, particularly DNA. A review of the studies on the process is presented, with the Maillard reaction described as the main mechanism by which reducing sugars interact with amino groups of proteins and nucleic acids, highlighting its importance to human health and activity. The author thoroughly examines the influence of glycation on the structure and function of proteins and DNA, focusing on its role in disrupting genomic stability and the accumulation of mutations, as well as its involvement in various pathologies and aging processes. The dissertation also reviews the mechanisms developed throughout evolution to protect biological macromolecules from glycation. Presenting the existing knowledge about DNA glycation and deglycation processes, the author logically justifies her further research. The literature review is rich in information, demonstrating that the author is highly knowledgeable in her field.

The objective of the dissertation is succinctly formulated: "TO IDENTIFY AND CHARACTERIZE THE DEGLYCATION ACTIVITY IN *ESCHERICHIA COLI* K12 RESPONSIBLE FOR THE REMOVAL OF FRUCTOSE-6-PHOSPHATE RESIDUES FROM DNA." The dissertation outlines 13 tasks, but the first 6 fully achieve the stated goal. The remaining tasks greatly expand the study to include the human enzyme and present structural studies related to the reaction mechanism catalyzed by phosphoglucose isomerase.

To achieve these tasks, the author employed a wide range of modern methods: biochemical, chromatographic, and electrophoretic techniques, immunoblotting, immunoprecipitation, quantitative protein and DNA concentration assays, enzymatic tests, bioinformatic analyses, and molecular dynamics simulations. The detailed and precise description of the methods makes the dissertation a valuable reference for researchers in the field. The results encompass a series of experiments systematically investigating the DNA deglycation activity of the glycolytic enzyme phosphoglucose isomerase (PGI) in *Escherichia coli* and human cells, revealing the enzyme's role in maintaining genomic stability.

Through chromatographic fractionation of cell lysates from *E. coli*, the author obtained fractions enriched in DNA deglycation activity. Using two-dimensional electrophoresis and protein sequencing from these samples, she identified PGI as the enzyme responsible for the observed DNA deglycation activity. This result is unexpected, as PGI is a known glycolytic enzyme, and its possible role in DNA deglycation represents a novel finding in the scientific literature.

After identifying PGI as a potential enzyme displaying DNA deglycation activity, the author explored its ability to bind to DNA. Using the gel-shift method for protein-DNA complex mobility, PGI exhibited slight mobility retardation in its complex with DNA (compared to free DNA), suggesting an interaction between the enzyme and DNA. Immunoprecipitation from bacterial cell lysates confirmed that PGI binds to DNA not only in vitro but also in vivo. These results suggest that PGI may play an active role in processes related to genomic stability maintenance. The author developed an enzyme assay for PGI's deglycation activity, using high-molecular-weight DNA glycated with glucose-6-phosphate (G6P), and found that the enzyme successfully recognizes double-stranded glycated DNA (DNA-NH-F6P) and catalyzes the removal of fructose-6-phosphate residues. Boteva performed kinetic analysis of the deglycation reaction and found that the enzyme's affinity for glycated DNA is similar to that of its natural substrate under physiological conditions. These results confirm the possibility of PGI performing a deglycation reaction on DNA. The physiological role of PGI in *E. coli* was also studied by comparing the frequency of spontaneous mutations in strains with and without an intact PGI gene. It was found that PGI-deficient strains exhibited a significantly higher mutation rate, indicating that PGI's deglycation activity is related to maintaining genomic stability.

The next stage of the research involves the human homolog of the enzyme phosphoglucose isomerase (hPGI). Through immunoblotting after subcellular fractionation and immunofluorescence, the author clearly demonstrates the presence of hPGI in the nuclei of human cells from the HEK293 and PC3 lines. This suggests that the enzyme may also participate in the DNA deglycation processes in human cells. Similar to the earlier experiments in bacteria, to confirm the DNA-binding activity of hPGI, Boteva conducted chromatin immunoprecipitation (ChIP) and found that hPGI binds to DNA in the nucleus. These interactions suggest that hPGI could act as a DNA deglycase in humans. It has been shown that all proteins that co-immunoprecipitate with hPGI are involved in maintaining genomic stability. Kinetic studies of hPGI revealed that both the bacterial and human enzymes have similar specific activities and kinetic parameters in their roles as isomerases and deglycases. The obtained results clearly indicate the functional similarity between the human and bacterial enzymes.

Further, the author conducts detailed bioinformatic analyses comparing the structural features of PGI from *E. coli* and other bacterial deglycases. The analysis of the spatial structures of PGI and FrlB from *E. coli* (predicted from the primary structure using the Google AlphaFold tool) shows overlap, despite the functional activity differences between the enzymes. Additionally, a nuclear localization signal was found in the isoforms of human hPGI, suggesting that the human enzyme participates in DNA repair.

Next, Boteva proceeds with research through molecular modeling and molecular dynamics. Using molecular docking methods, she shows in silico interaction between DNA-NH-F6P and the active site of PGI. Docking simulations demonstrate that the substrate is stably positioned in the catalytic center of PGI, and molecular dynamics simulations show that the complex between PGI and glycated DNA remains stable, allowing the deglycation reaction to be catalyzed. Metadynamics simulations confirm that the movement

of the substrate toward the catalytically active position is energetically favorable, providing additional evidence for PGI's deglycation function.

Overall, the experiments conducted show, beyond resonable doubt, that PGI, both in bacteria and in humans, not only participates in metabolism but also in the maintenance of genomic stability through the removal of glycated DNA adducts. The results reveal a new aspect of PGI's physiological role and open up extensive possibilities for research linking sugar metabolism and genomic stability, given the importance of these areas for human pathologies and aging.

The "Results and Discussion" chapter concludes with a subchapter entitled "Discussion," where the author thoroughly and competently discusses the dissertation results in the context of known global literature, emphasizing both the molecular mechanism of PGI's amadoriase activity and the enzyme's physiological role.

Conclusion: Elitsa Hristova Boteva's dissertation represents a rare example of comprehensive research on a clearly defined and important question in cellular physiology. The work reflects the author's pursuit of a better understanding of the details of the studied process. This neccessitated the successful application of a wide range of modern experimental methods and bioinformatics techniques. This pursuit of thoroughness and the attention to detail has allowed the author to delve deeply into the topic, exceeding what is typical for such work. Despite its large volume, the dissertation is a pleasure to read, thanks to both the excellent experimental work and the author's superb writing style and the logical coherence of the dissertation. Even half of this work would be more than sufficient for a doctoral defense. Considering all of the above, I confidently recommend that the esteemed scientific jury award the educational and scientific degree "Doctor" to the author. In conclusion, I believe that Elitsa Boteva will continue to work at such a remarkable level and wish her a long and successful career in science.

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