Final Report

Biomachinery of uridine isomerization: structure and catalysis of box H/ACA pseudouridine synthase.

In this project we planned to study the elaborate reaction mechanism of the complex protein/RNA system responsible for the chemical modification of uridine, affording the most abundant and significant post-transcriptionally modified RNA base, pseudouridine. We succeeded in uncovering that certain dyskerin mutations cause pseudouridylation defect of box H/ACA pseudouridine synthase, the physiological effects of which can be best described as a ribosomopathy – the shortcoming of protein synthesis, due to the imperfect assembly of the ribosome. We showed that both distant mutations and those within the active site disrupt the catalytic potential by removing an Asp sidechain from the proximity of the substrate uridine, and thus abolish the specific binding mode of the wild type (wt) complex. We also demonstrated, that the uridine to pseudouridine transformation can only be initiated from this specific, strained and bent binding conformation, describing a new mechanisms for the catalytic reaction.

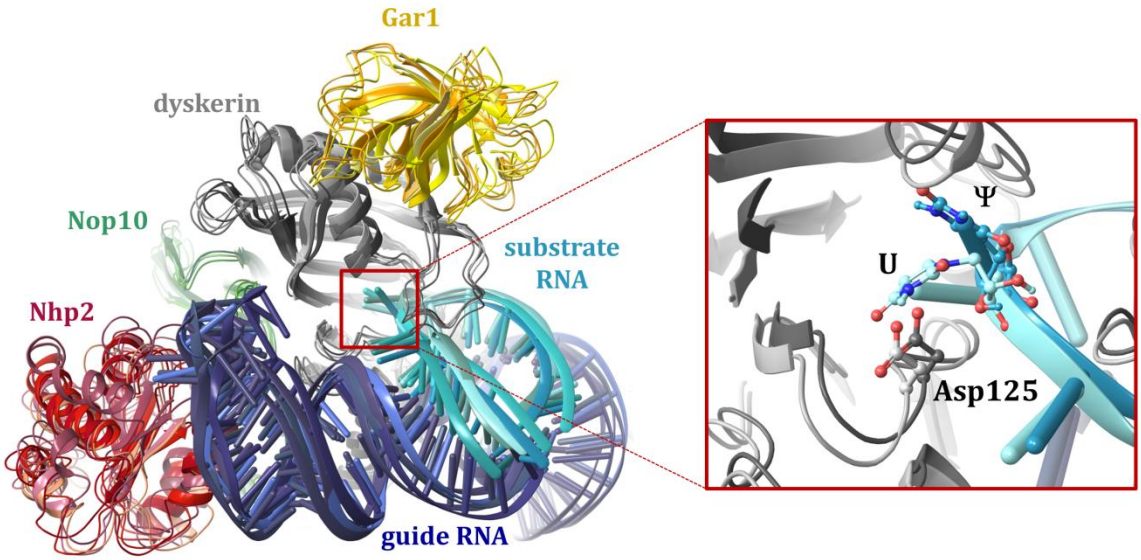
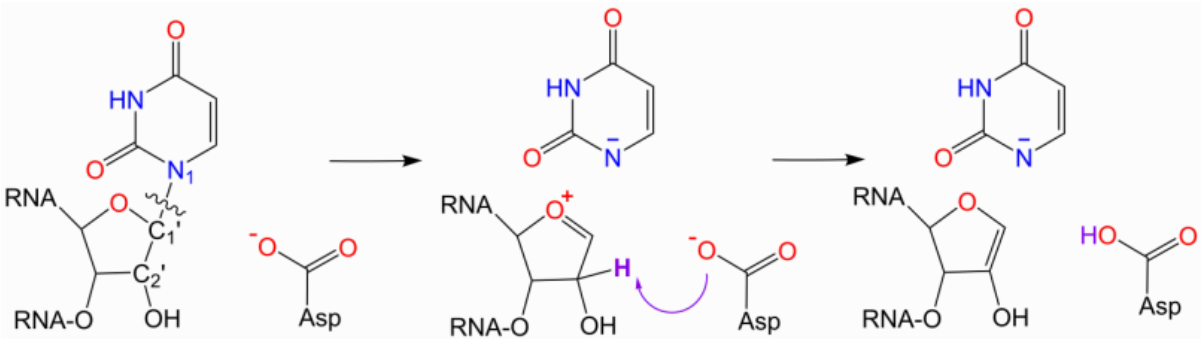


Figure 1. The box H/ACA pseudouridinase complex formed by four proteins and 2 RNA chains (most prevalent structure of the MD simulations of the human enzyme in the reactant and product states of the wt and its *dyskerin.*D125A, D125N, E206K mutants). The inset shows the distortion of the substrate uridin (U) enforced by the enzyme compared to the upright position of the pseudouridine (ψ) product.

During the first year we built a homology model of the human pseudouridine synthase enzyme in the wt and disease causing mutant form, where the latter carried a mutation of dyskerin (E206K) that alters its interaction with Nop10, the neighboring protein component of this extended biomachinery formed by four proteins and 2 RNA chains. During the second year, a corresponding mutation of Nop10 (T16M) on its surface facing dyskerin was located and shown to cause a phenotype quite similar to that discovered by our cooperating partners (K. Tory and his coworkers, Semmelweis University). Therefore the structural effects of this mutation were also investigated in detail. In our third and fourth year the measured differences in binding affinities between dyskerin and Nop10 in the WT and mutant systems were successfully rationalized by MD simulations that also provided an H-bonded network connecting the active site and the mutational site. Active site changes taking place upon either mutation were found significant enough to explain the pathogenicity of the mutant systems. Results indicate that the mutations remove the catalytic Asp from the proximity of the uridine substrate, where it should participate in the proton abstraction step of the reaction (according to

suggestions found in the literature) or provide energetic boost of the reaction by the distorted coordination of the substrate uridine (according to our own QM/MM results) – thus directly hinder the pseudouridylation reaction.

In the following years we initiated QM/MM MD calculations for obtaining a realistic reaction path for the catalytic reaction of the pseudouridinase (PSU) complex. The various reaction mechanisms proposed in the literature were examined using small model systems and QM calculations, to conclude that only the glycal mechanism – initiated by the C1'-N1 bond breakage between the sugar and the base of the substrate uridine, followed by its C2' deprotonation –proved to be a real possibility for the pseudouridylation reaction.



Scheme 1. The first two steps of the glycal mechanism

Relying on the MD-derived equilibrium ensembles of reactant and product states of the wt PSU complex and its *dyskerin*.D125A, D125N, E206K mutants, QM/MM models were built to study the reaction profile including the surrounding protein- and RNA-molecules and the solvent. The free-energy profile was refined using umbrella sampling in DFTB3/Amber14 QM/MM MD calculations. This methodology afforded a very favorable, 14 kcal/mol, energy barrier for the first step of the reaction that is finally in accordance with the experimental results – but only in the case of the wt complex. Based on the results we proposed a completely new mechanism for the first step of the reaction: our findings suggest that the strain caused by the distorted binding of the targeted uridine of the substrate RNA chain, supported by the surrounding electrostatic pattern, initiates the cleavage of the bond and the subsequent formation of the C1'-C5 association. We were able to demonstrate that the highly strained conformation only appears in the wt reactant state complex carrying a uridine in the substrate-binding position – in all mutants and in case of the pseudouridine-containing product complex the base is in a relaxed upright conformation. We also showed that the final proton transfer steps of the reaction will only proceed if the catalytic pocket opens up and water is allowed inside – a movement also detected in the MD simulation of the product state. The revised manuscript describing these results is currently under review at *ACS Catalysis*.

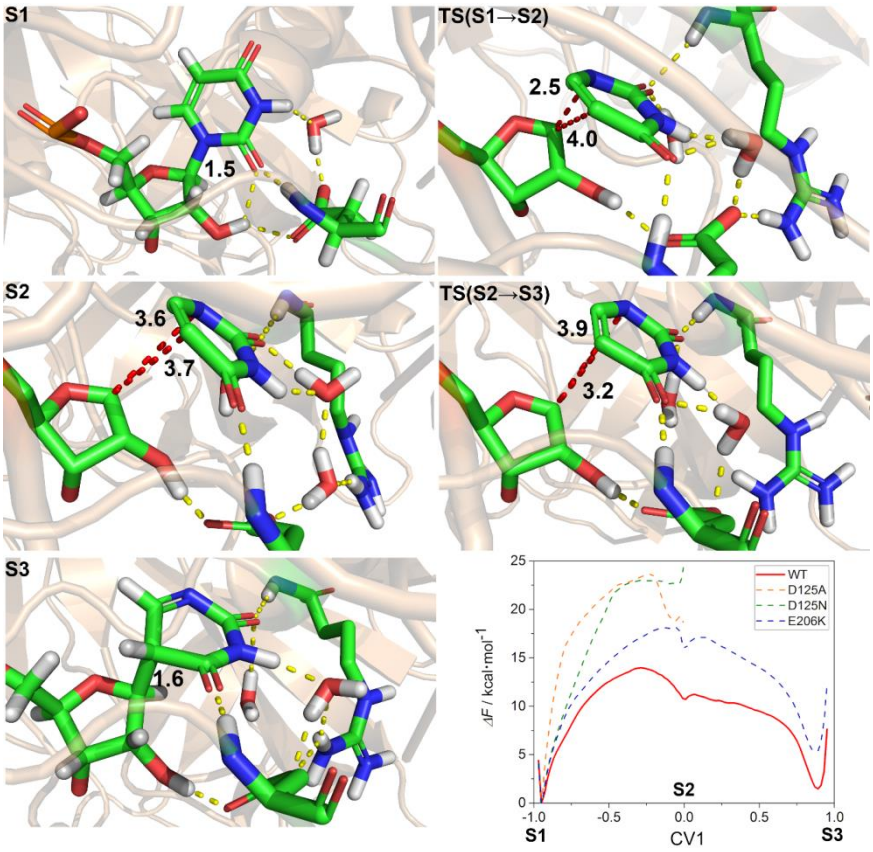


Figure 2. The base dissociation, flip and rebind steps of the catalytic reaction of human box H/ACA psueudouridinase

We also discovered that the association of the guide RNA and the rest of the pseudouridylation complex is virtually non-sequence dependent – only about 5% of the H-bonds formed between the guide RNA and the proteins of the complex is between the nucleobases of the catalytic region and the proteins of the complex, the rest of the associations are either far from the site of pseudouridylation or formed by the sugar-phosphate backbone of the guide. Accumulating evidence suggests that pseudouridylation of mRNAs can result in the incorporation of alternative amino-acids and the read-through at premature stop codons (PTCs). As a significant proportion (~10%) of pathogenic human mutations are attributed to PTCs, targeted pseudouridylation provides a potential clinically relevant strategy to correct these mutations – if guide RNAs can be designed for the incorporation of the targeted mRNA segments into the pseudouridylation machinery. Demonstrating that there are practically no limitations on the sequence of the guide RNAs that the pseudouridinase complex can coordinate and process, opens the possibility of its application in RNA editing and its future therapeutic use (these results were presented at a Keynote symposium on RNA Editing and Modifications).

The lessons we learned from the extensive MD and QM/MM study of the PSU complex were successfully applied to the study of further similar systems, where distant mutations that do not cause drastic conformational changes exert dramatic influence over catalytic efficiency. In collaboration with the NMR group of the Laboratory of Structural Chemistry and Biology (ELTE), we identified the key dynamical changes that drive the functionally different behavior of the wt and mutant oncogenic KRas enzymes, despite the fact that mutations cause little change in the overall structure of the system. Using QM/MM calculations we could describe the effect of

the subtle changes on the catalytic mechanism of this protein – that is responsible for over 20% of all human cancers. Another protein, where hetero- and homomultimerization – just as seen in case of pseudoridinases –is a prerequisite of function, is podocin of the glomerular filtration system of the kidney. Over the last couple years (in collaboration with the I. Department of Paediatrics, Semmelweis University), we have been able to show that disease casing mutations alter the dimerization patterns and affinity of podocin and thereby also affect its association with nephrin, the long chain-like molecules that weave the net functioning as the actual molecular filter of the kidney. We have also been able to confirm that the even spacing of the nephrin-net is regulated by the rigorous dimerization and oligomerization of podocin – the reason why its mutations are so physiologically severe.

The computational resources obtained (Schrödinger Suite) allowed for further participation in projects concerning tumor suppressant p53, a unique model for the aggregation process based on the configuration dependent phase transfers of a glucose-amine derivative, binding of gas molecules to heme-proteins and yet another huge protein complex (GINS) where the effect of mutations on inter-protein association and reactivity could be studied.

We have requested extension of the time period of this project due to the pandemic, which caused delays in our workflow for various reasons. Beside that fact that we were forced into quarantine which slowed down both experiments and calculations, immense time was taken up by the need to redesign out teaching curriculum and practice, to adept it to the online world. But also, and more importantly, we had an opportunity to participate in the discovery process of a new medication for treatment of the early stages of coronavirus in cooperation with Richter Nyrt and other academic groups, which was patented as P2100038 ((2021.02.03): Mutated Recombinant Ace2-Fc Fusion Proteins for the Treatment of CoVID-19 Infections) and is currently being tested in animal models, having been shown quite effective in cell-based essays.

The work carried out within this project was presented in 17 published papers (plus an additional one, that - in its revised form - is currently being evaluated in *ACS Catalysis*), and at 18 conference appearings (either oral presentations or posters).

The papers of the final year of the project:

The structure-derived mechanism of box H/ACA pseudouridine synthase offers a plausible paradigm for programmable RNA editing D. J. Kiss, J. Oláh, A. Stirling, G. Tóth, M. Varga, D. K. Menyhárd, Gy. G. Ferenczy (revised manuscript under evaluation at *ACS Catalyis*)

Pseudouridylation defect due to DKC1 and NOP10 mutations causes nephrotic syndrome with cataracts, hearing impairment, and enterocolitis E. Balogh, J. C. Chandlerc, M. Varga, M. Tahounc, D. K. Menyhárd ….and K. Tory (2020) Proc. Natl. Acad. Sci. USA, , 117, 15137–15147.

Tumor-Suppressor p53TAD1–60 Forms a Fuzzy Complex with Metastasis-Associated S100A4: Structural Insights and Dynamics by an NMR/MD Approach. E. F. Dudás, Gy. Pálfy, D. K. Menyhárd, F. Sebák, P. Ecsédi, L. Nyitray and A. Bodor (2020) Chembiochem., 21(21), 3087-3095

Configuration-Controlled Crystal and/or Gel Formation of Protected d-Glucosamines Supported by Promiscuous Interaction Surfaces and a Conformationally Heterogeneous Solution State. A. Kapros, A. Balázs, V. Harmat, Adrienn Háló, L. Budai, I. Pintér, D. K. Menyhárd, and A. Perczel (2020) Chem. Eur. J. 26, 11643-11655.

Structural impact of GTP binding on downstream KRAS signaling. D. K. Menyhárd, Gy. Pálfy, Z.Orgován, I. Vida, Gy. M. Keserű, A. Perczel (2020) Chem. Sci.,11, 9272-9289.

Dynamically encoded reactivity of Ras enzymes: opening new frontiers for drug discovery Gy. Pálfy, D. K. Menyhárd, A. Perczel (2020) Cancer Metastasis Rev. doi: 10.1007/s10555-020-09917-3.

Tissue-Specific Requirement for the GINS Complex During Zebrafish Development. M. Varga, K. Csályi, I. Bertyák, D. K. Menyhárd, ….., S, W. Wilson (2020) Front. Cell Dev. Biol. 8: 373

Gas Sensing by Bacterial H-NOX Proteins: An MD Study. A. M. Rozza, D. K. Menyhárd, J. Oláh (2020) Molecules 25, 2882

Conferences in the final year of the project:

The structure-derived mechanism of box H/ACA pseudouridine synthase offers a plausible paradigm for programmable RNA editing. D. J. Kiss, D. K. Menyhárd, J. Oláh, A. Stirling, G. Tóth, M. Varga, Gy.G. Ferenczy. Keystone Symposia: RNA Editing and Modifications: From Biology to Therapy (online conference: Sept. 30 – Oct. 2, 2020)

Mutations in dyskerin (dkc1) are associated with growth impairment and defective cell cycle in the zebrafish (Danio rerio) R. Hamar, N. Borbély, M. Penc, D. Kaszás, E. Balogh, R. Légrádi, D. K. Menyhárd, K. Tory and M. Varga. Keystone Symposia: RNA Editing and Modifications: From Biology to Therapy (online conference: Sept. 30 – Oct. 2, 2020)

Podocin regulates the size of the glomerular pore. M. Kétszeri, V. Antal, G. Schay, A. Ungvári-Veres, K. Pászty, M. Kellermayer, D. K. Menyhárd, K. Tory. 57th Nephrology Virtual Congress, June 6-9, 2020