DISCIPLINE COUNCIL FOR CHEMICAL SCIENCES

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ANALYSIS OF MOLECULAR ASPECTS OF PROTEINS REGULATION CONSIDERING WATER MOLECULES AS A POTENTIAL MEDIATOR IN INTERMOLECULAR INTERACTIONS

EXTENDED ABSTRACT

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Scope of the doctoral thesis

The unprecedented importance of the role of water in various biological processes was the main motivation to tackle this topic during the doctoral studies. The doctoral dissertation presents the results of several works related to modelling the dynamics of water molecules in biological systems, resulting from the use of dedicated software and computational methods. Results presented in this thesis are related to the analysis of the various functions performed by water molecules in proteins. Specifically, they concern three areas of application: drug design, protein regulation and engineering, as well as studying enzymatic reaction.

Aims of the doctoral thesis

Four main objectives of the doctoral thesis were defined. They were as follows:

1. To survey available computational tools that incorporate water molecules for studying macromolecules' properties and to participate in the development of a new version of software enabling the structural and functional analysis of macromolecules from the ‘intramolecular voids’ perspective.
2. To demonstrate applications of water and (co)solvent molecules in computational drug design-related studies.
3. To explore applications of water molecules in protein engineering and macromolecule regulation.
4. To characterise the role of water molecules in proteolytic cleavage enzymatic reaction, a process related to the regulation of a signalling pathway.

To fulfil the first objective, I participated in the preparation of the review article (**Paper 1**) [1] that summarised information about various software and tools that can be incorporated for studying the macromolecules’ properties using water molecules. Moreover, I was part of the team which worked on and developed the new version of the AQUA-DUCT software (AQ 1.0), dedicated to performing the structural and functional analyses of macromolecules using a novel, intramolecular voids perspective (**Paper 2**) [2]. To accomplish the second goal, I was involved in studying and describing the applications of water and (co)solvent molecules in computational drug design-related studies for the following molecular targets: the main protease of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2 Mpro) (**Paper 3**) [3], the SARS-CoV-2 Mpro and a panel of selected proteases (**Paper 4**) [4],

and the human soluble epoxide hydrolase (hsEH) (**Paper 5**) [5]. To achieve the third objective, I participated in studying the soluble epoxide hydrolase (sEH) subfamily and describing the structure-function relationship between sEH structure and their tunnel network (**Paper 6**) [6]. I was involved in comparing geometry-based and small-molecule tracking methods for tunnel identification (**Paper 7**) [7]**.** I also examined the identified tunnels in terms of their evolution (**Paper 8**) [8]. Regarding the fourth goal, firstly, I outlined the problem with studying the proteolytic cleavage reaction in Toll-like receptors (TLRs) (**Paper 9**) [9]. Then, I participated in characterising this process in the TLR8 with a particular emphasis on the role of water molecules in the course of this reaction. The results of the later study have been published as a preprint (**Preprint 1**) [10].

Summary of the doctoral research and personal contribution

Analysis of macromolecule structure, properties, and functions with the use of water molecules

In **Paper 1** entitled "Applications of water molecules for analysis of macromolecule properties", available computational methods that employ water molecules to analyse the properties and structural dynamics of macromolecules were reviewed. The article was divided into three subsequent parts. The first part was focused on describing software for analysing protein hydration, while the second section was dedicated to providing an overview of tools for detecting water sites and analysing ligand-binding events. Finally, the third part was aimed at reviewing software for tunnel detection and various transportation phenomena. My contribution was in gathering information and providing descriptions regarding the applicability and functionality of software reviewed in the second and third parts of the article. Overall, I participated in organising data, writing the manuscript, reviewing and editing the final version, and providing answers to the reviewers' comments.

In **Paper 2** entitled "AQUA-DUCT 1.0: structural and functional analysis of macromolecules from an intramolecular voids perspective", the new functionalities of the AQUA-DUCT software were presented. In particular, the focus was on two types of analyses that one can perform using the presented tool - advanced small-molecule tracking and local-distribution analysis. I took part in the preparation and development of the new version of the software,

mainly by testing new features that were added to drivers that use *aqueduct* module to perform relevant analyses. I was working with the main developer of the software on the optimisation of the *pond* driver which enables the local-distribution analysis and facilitates the detection of pockets and hot-spots within the macromolecule's structure (**Figure 1**). In addition, I was testing distinct modes of analysis that have been implemented in the new version of AQ. I was working on the following case studies: human soluble epoxide hydrolase, potato soluble epoxide hydrolase, and haloalkane dehalogenase LinB. I was involved in writing the manuscript, preparation of figures and also in reviewing and editing the final version, as well as in providing answers to the reviewers' comments.

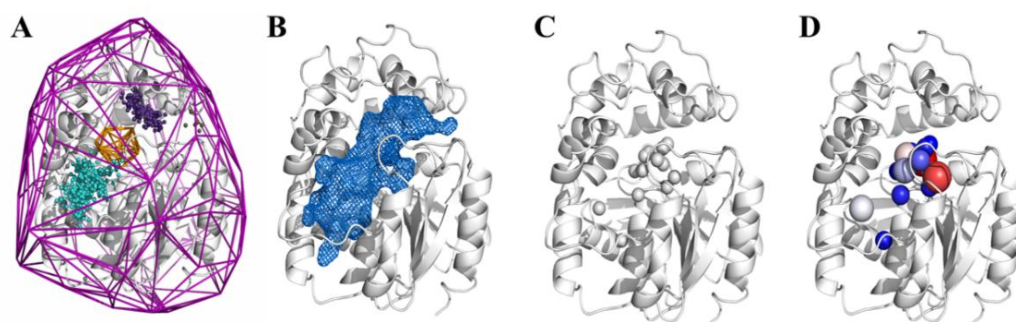

Figure 1 An example of local-distribution analysis performed with AQUA-DUCT 1.0 software. **(A)** Visualisation of clusters of inlets. **(B)** Visualisation of pockets identified within protein's interior. **(C)** Visualisation of regions with a higher density of water molecules (hot-spots). **(D)** Visualisation of hot-spots reflecting their density. Figure adapted from [11] with some modifications.

Applications of water and (co)solvent molecules in computational drug design-related studies

The first application of water and (co)solvent molecules in drug design-related studies incorporated the work on the main protease of SARS-CoV-2 – SARS-CoV-2 Mpro. The results of analyses were gathered in **Paper 3** entitled "SARS-CoV-2 Mpro as a challenging molecular target for small-molecule inhibitor design". In **Paper 3**, I share the first authorship with Karolina Mitusińska. I was responsible for running all mixed-solvent MD simulations and their further analysis with AQUA-DUCT 1.0 software, including the analysis of the intramolecular voids and hot-spots. Also, I was involved in conducting the same analysis for classical MD simulations. Together, we made a comparison of the crystal structures of Mpros from SARS-CoV-2 and SARS-CoV and performed an analysis of the plasticity of their binding cavities. I was also involved in the analysis of the potential mutability of SARS-CoV-2 Mpro. Additionally, I compared the space occupied by covalently bound

fragments in the active site cavity with an accessible volume computed by AQUA-DUCT. Overall, I was involved in writing the manuscript, preparation of figures and also in reviewing and editing the final version, as well as in providing answers to the reviewers' comments.

In **Paper 3**, classical MD simulations with water molecules as molecular probes were used to provide a detailed picture of the SARS-CoV-2 and SARS-CoV main proteases' interior dynamics. The small-molecule tracking approach was employed to assess the accessibility of the active site pocket, and a local-distribution approach to provide information about the solvent distribution within the proteins' interior. To identify potential binding/interacting sites in Mpro structures, mixed-solvent MD simulations with six different co-solvents were performed. The distribution of global hot-spots from different co-solvents revealed specific interactions with particular regions of the analysed proteins. The active site region and the region involved in the Mpro dimerisation showed the highest density of hot-spots. Insight from the local hot-spots distribution underlined the differences in binding sites' plasticity (**Figure 2**).

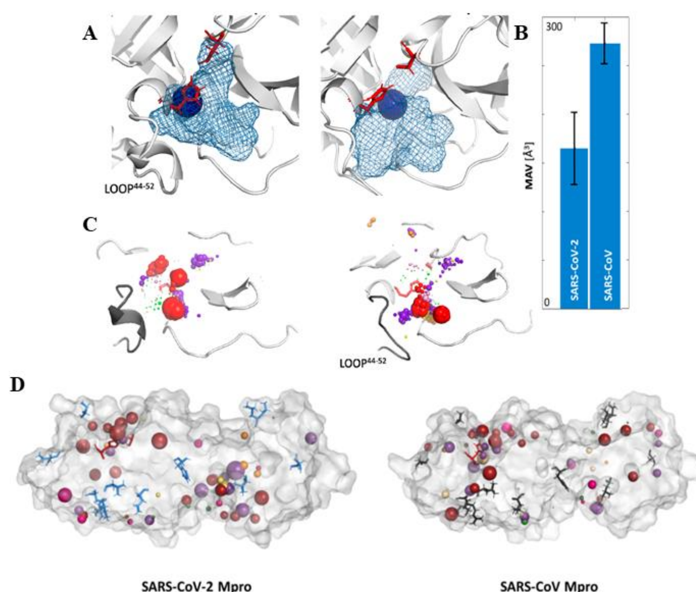

Figure 2 Differences in binding cavities and within the entire structures for SARS-CoV-2 and SARS-CoV main proteases. **(A)** and **(B)** Maximal accessible volumes for the solvent molecules in the binding cavities of apo structures of SARS-CoV2 and SARS-CoV Mpros together with the position of the identified water hot-spots with the highest density **(C)** Localisation of co-solvent hot-spots identified in the binding cavity of both enzymes. **(D)** Localisation of co-solvent hot-spots identified within the entire structures of both enzymes. Figure adapted from **Paper 3** [3] with some modifications.

In **Paper 4** entitled "Computational Selectivity Assessment of Protease Inhibitors against SARS-CoV-2", MD simulations protocols, together with molecular docking, and toxicity profiling were used to assess the selectivity of non-covalent inhibitors of SARS-CoV-2 Mpro

against eight different proteases and 16 anti-targets. In **Paper 4**, I share equal authorship with André Fischer, Manuel Sellner and Karolina Mitusińska. We were all involved in the selection of the panel of proteases for carrying out the research. Together with Karolina Mitusińska, I was involved in the analysis of the similarity of the selected proteins, especially in the context of their active site. Then, we performed all the classical MD simulations together with the water molecules tracking and hydration sites identification. Additionally, I was involved in the analysis of the co-solvent sites, identified based on the mixed-solvent MD simulations carried out by colleagues from the University of Basel. Altogether, we assessed the selectivity of analysed proteases, taking into account different perspectives. I was involved in writing the manuscript, preparation of figures and also in reviewing and editing the final version, as well as in providing answers to the reviewers' comments.
The set of analysed proteins included the following proteases: SARS-CoV-2 Mpro, SARS-CoV Mpro, caspase-3, factor Xa, cathepsin G, ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1), prostasin, thrombin, and chymase. Overall, a comprehensive analysis of the selectivity of a set of proteases from different perspectives was performed, including sequence and active site similarity, the location of hydration and small-molecule hot-spots, as well as molecular docking and toxicological profiling. Taking into consideration all the analysed factors, the highest potential for off-target binding of SARS-CoV-2 Mpro inhibitors was predicted for factor Xa, SARS-CoV Mpro and cathepsin G, but also in some aspects for chymase, and UCHL1. A lower probability of off-targeting was estimated for prostasin, and thrombin, and the lowest for caspase-3.

Another example of the application of water molecules in drug design-related studies was presented in **Paper 5** entitled "Computational insights into the known inhibitors of human soluble epoxide hydrolase". In **Paper 5** all deposited hsEH–ligand complexes were analysed to gain insight into the binding of inhibitors. Also, the architecture of the hsEH hydrolase domain was analysed, mostly by incorporating the analysis of solvent transportation and internal voids' dynamics. In this article, I am the first author and I carried out all the analyses for known inhibitors co-crystallised with hsEH. I performed the analysis of the interactions, including clustering of binding residues and inhibitors, I ran MD simulations and accomplished the identification of tunnels and inner voids within the hsEH structure. I was involved in writing the manuscript, preparation of figures, in reviewing and editing the final version, as well as in providing answers to the reviewers' comments.

The results indicated that, in the case of hsEH, the interactions with active site residues and their surrounding are not vital for successful inhibitor design. It was proposed that besides occupying large hydrophobic moiety, small inhibitors could be positioned on the border of the buried and surface-exposed residues and might benefit from residues donating functional groups, which are essential for increasing the solubility of the compounds. Additionally, analysis of all deposited hsEH–inhibitor complexes indicated that the inhibitors do not fully occupy the available internal cavity and that there is still some unused space that could host novel inhibitors. The MD-simulation-based analysis provided information about additional potential locations (entries/exits to tunnels) where novel inhibitors could bind. The possibility of targeting the potential novel binding sites was also confirmed through running additional mixed-solvent MD simulations [12]. In general, three novel potential binding sites were indicated, which were further used as targets for investigating the binding of novel inhibitors.

Applications of water molecules in protein regulation and engineering

Analyses incorporating water molecules in protein regulation and engineering were performed for enzymes from the soluble epoxide hydrolase subfamily. Due to the quite a diverse network of tunnels in members of the sEH subfamily, those enzymes turned out to be a very good case study to characterise their tunnel network and draw conclusions that may be useful, e.g. in the context of protein regulation and engineering, also for other families.

In **Paper 6** entitled “Structure-function relationship between soluble epoxide hydrolase structure and their tunnel network” (and further Corrigendum to this article), the available structures of soluble epoxide hydrolases were examined and a comprehensive analysis of their tunnel network was conducted. By using water molecules as probes, it was possible to investigate the internal voids of sEHs and gain a deeper understanding of their architecture. This analysis allowed to elucidate the structural characteristics of the tunnel network in sEHs.

In this article, I was mainly involved in analyses of the flow of water molecules for all the analysed structures, particularly the determination of the appropriate parameters for detecting tunnels and their subsequent characteristics. I also participated in a general comparison of the sEH subfamily members by analysing their structural features. I was involved in data organisation, and partially in writing and reviewing the manuscript, as well as responding to the reviewers’ comments.

In **Paper 6**, the structural features of the selected members of the soluble epoxide hydrolase subfamily were examined. Based on the sequence and structural similarities, the analysed enzymes were we classified into three groups. Group I included mammalian and fungal sEHs, group IIa consisted of plant sEHs, and group IIb included bacterial and thermophilic sEHs. The analysis of water molecule movements provided insight into the tunnel network in different regions of sEHs. Based on the tunnel usage, it was possible to indicate that some members predominantly utilise both Tc/m and Tm1 tunnels (representatives of mammals and fungi), while others rely rather on one of these tunnels - Tc/m (representatives of bacterial and members from an unknown source) or Tm1 (representatives of plants), respectively (**Figure 3**). These distinct patterns were in agreement with the analysis of structural features, indicating a relationship between the structure and function in members of the soluble epoxide hydrolase subfamily. Also, it was possible to find out that the structural and dynamic features of proteins translate into the shape, size and utility of individual tunnels and, consequently into the preference of recognised substrates and further catalytic efficiency.

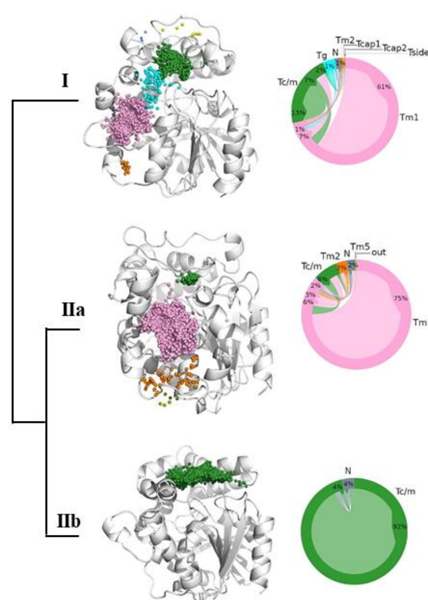

Figure 3 Results of the identified entries/exits to the tunnels in the selected members of soluble epoxide hydrolase (sEH) together with the intramolecular flow plot. The results are presented for three selected members – from Group I for *Homo sapiens* (hsEH), from Group IIa for *Solanum tuberosum* (StEH1), from Group IIb for *Bacillus megaterium* (bmEH), representing different patterns of tunnel utilisation. Figure adapted from **Paper 6** [6] with some modifications.

Members of sEHs were a good set of data that could be used to conduct a detailed comparison of different approaches aimed at identifying tunnels in proteins. Therefore, such a comparison was made and the results were published in **Paper 7** entitled “Geometry-based versus small-molecule tracking method for tunnel identification: benefits and pitfalls”. Two distinct

tools were employed to represent different approaches: AQUA-DUCT 1.0 for the small-molecule tracking approach, and the commonly used CAVER 3.0 PyMOL plugin along with CAVER 3.02 for the geometry-based approach. In this article, I share the first authorship with Karolina Mitusińska. We conducted the analyses with the following division: I was involved in identifying tunnels in MD simulations using the small-molecule tracking approach, while Karolina Mitusisńska focused on the application of geometry-based approach. Together we also identified the tunnels in crystal structures using a geometry-based approach. Moreover, I was involved in refining the method for comparing tunnels found in crystal structures with those identified during MD simulations. I was involved in writing the manuscript, preparation of figures, reviewing and editing the final version, as well as in providing answers to the reviewers' comments.

In **Paper 7**, the goal was to mimic a typical workflow used for tunnel identification in proteins. Therefore, the study started with the simplest geometry-based analysis of previously selected sEHs crystal structures. Then, the analysis was expanded, to include the information from the MD simulations and compare both geometry-based and small-molecule tracking methods. Such a comprehensive approach allowed for a comparison of results obtained with distinct methods, highlighting their respective advantages, limitations, and potential biases. Overall, it was highlighted that MD simulations may offer a more comprehensive understanding of protein tunnel networks, and that the small-molecule tracking approach complements the geometry-based methods.

The culmination of the research on tunnels in sEHs was the analysis of their evolution. The results were published as **Paper 8** entitled "Evolution of tunnels in α/β-hydrolase fold proteins – What can we learn from studying epoxide hydrolases". In **Paper 8**, I again share the first authorship with Karolina Mitusińska. In this article, we presented the entire pipeline of evolutionary analysis of tunnels (and also other structurally important compartments) in the sEHs subfamily. Specifically, I performed the evolutionary analysis of the residues making up particular compartments and all tunnels in each of the selected member of sEHs. I evaluated the overall variability for both the referential compartments and tunnels. Besides, I participated in providing characteristics about the general evolutionary analysis of tunnels and a detailed analysis of the selected cases which culminated in proposing the perforation mechanism of the tunnel formation that could be applied as a strategy for *de novo* tunnel design. I was involved in writing the manuscript, preparation of figures, reviewing and editing the final version, as well as in providing answers to the reviewers' comments.

The evolutionary analysis for reference compartments revealed that the active site amino acids were conserved, while surface residues were the most variable. This confirmed the well-known observation that amino acids comprising the macromolecule surface evolve faster, while the active site residues remain well-preserved. Generally, buried residues showed lower variability. The cap-loop and NC-loop compartments were classified as a variable in all sEHs, except for the NC-loop in CH65-EH. α-helices and loops were also variable, except for hsEH and StEH1 where the variability of loops was not statistically significant. Β-strands were conserved in all analysed proteins, except for msEH. Both main and cap domains were classified as variables.

For the tunnel evolution study, it was hypothesised that tunnels should be rather conserved structure features, but equipped with some variable parts. The results confirmed that almost all analysed tunnels could be considered conserved. To get better insight into the location of the variable/conserved residues along tunnels, additional analyses were performed. For that, three different tunnels identified in various sEHs were selected – Tc/m tunnel of hsEH and the Tm1 tunnel of StEH1 which were identified as the most common tunnels in sEHs, and the Tc/m_back tunnel of bmEH as the case of a tunnel which was previously engineered. Also, these tunnels were selected because they exhibited different distributions of the entropy values. All the findings led us to propose a mechanism for tunnel formation. It was hypothesise that new tunnels can appear through mutations occurring not only on the protein surface but also at the border of large cavities, affecting surface cavities. In detail, mutations in variable residues can spontaneously drive the evolution of active site accessibility through surface perforation or the joining of internal cavities (**Figure 4**). Such a mechanism can be adapted for enzyme modification and can significantly improve enzyme performance by e.g. separating substrate/product transport pathways from water delivery pathways. Thus, identifying residues prone to causing such an effect can be valuable in protein re-engineering processes.

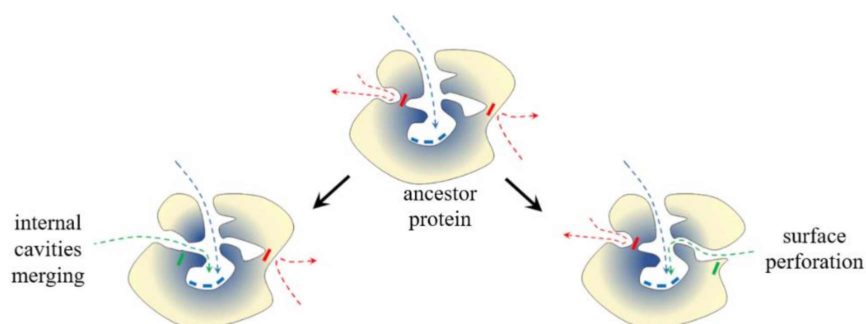

Figure 4 Schematic representation of the proposed theory for tunnel evolution – 'perforation model'. The first possibility shows the appearance of a new tunnel as a result of the merging internal cavities, while the second possibility shows the evolution of a new tunnel as a result of surface perforation. Figure adapted from **Paper 8** [8] with some modifications.

Roles of water molecules in the enzymatic reaction

During my doctoral studies, I also focused on studying the behaviour of more complex biological macromolecules, such as transmembrane proteins. Specifically, I was investigating Toll-like receptors (TLRs) - biomolecules that play an important role in the functioning of the immunity. Since I wanted to use computational methods to study the regulatory mechanisms of TLRs, it was necessary to summarise research performed so far and find out what has been achieved and what remains a challenge. With that, **Paper 9** entitled "Recent Advances in Studying Toll-like Receptors with the Use of Computational Methods" was published. In this article, TLRs were reviewed with a particular focus on both their function and mechanism of action. I am the first author of this paper. I performed the vast majority of the literature revision and organised all the data. I was writing the manuscript, reviewing and editing the final version, as well as providing answers to the reviewers' comments.
In this article, several areas in TLR research that require further development were identified. One of them was the need to investigate the proteolytic cleavage reaction in some members of the TLR family. Considering that the presence of water is crucial in the proteolytic cleavage reaction, I proposed to analyse the role of water in the TLR-protease system, within the entire reaction cycle. Specifically, I wanted to focus on investigating the behaviour of water molecules within the reaction site. TLR8 was selected for the investigation since it was confirmed that the cleaved form of the receptor is essential for proper functioning. Also the information suggesting the involvement of furin protease in this process was used.

The results of analyses were deposited as a **Preprint 1** "The proteolytic cleavage of TLR8 Z-loop by furin protease - molecular recognition, reaction mechanism and role of water molecules". In this article, I am the first author and I performed the majority of analyses related to the prediction of the TLR8-furin complex, analysis of the dynamics of the complex and interaction network (MD simulations for each reaction species), and analysis of the reorganisation of water molecules (AQUA-DUCT calculations). I was involved in writing the manuscript, preparation of figures, as well as the overall data organisation.
For each reaction species, reactant, intermediate states, and product (RE, INT1-INT3, and PROD, respectively), I performed MD simulations to analyse the complex dynamics, interactions network, and the reorganisation of water molecules. Analysis of the interaction network among residues from TLR8, furin protease and solvent molecules indicated that the general distribution of these interactions may differ between individual reaction steps.

For instance, significant differences in water molecule distribution at various stages of the reaction were noticed. At the very beginning, there were almost no water molecules present in the vicinity of the reaction site. As the reaction progressed, solvent molecules first occupied the reaction site and furin's interior, and then moved towards the TLR8-furin interface (**Figure 5**).

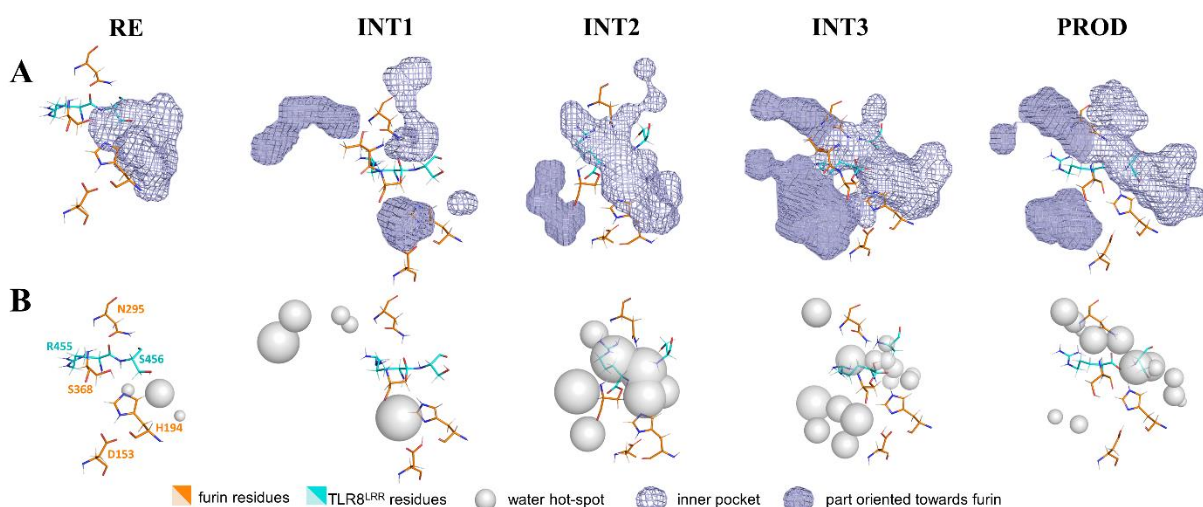

Figure 5 Analysis of water molecules reorganisation within the entire cycle of the proteolytic cleavage of the TLR8 Z-loop by furin protease. **(A)** Visualisation of the internal pockets penetrated by water molecules within the reaction site. **(B)** Localisation of the identified high-density water hot-spots. Figure adapted form **Preprint 1** [10] with some modifications.

The catalytic role of water in the reaction (for INT2) was confirmed by analysing the local distribution of solvent molecules in the analysed system. Nevertheless, additional potential roles of water molecules in other steps of the reaction were also proposed. For instance, water molecules could potentially stabilise the high-energy tetrahedral species INT1 and INT3. While analysing the MD simulations for these species, in some repetitions, it was possible to observe water molecules close to the negative charge developed on the R455 oxygen atom from the TLR8 cleavage site. These water molecules could assist or even take over the stabilising function from residues constituting the oxyanion hole. Moreover, the identified water hot-spots in the RE, INT3, and PROD species suggest that water molecules might act as a proton shuttle between furin's catalytic histidine and other residues. It was hypothesised that this water-mediated proton transfer could open up alternative reaction pathways. Finally, the observation of water movement towards the TLR8-furin interface might indicate the involvement of solvent molecules in the dissociation process of these macromolecules. An increased presence of water molecules between these macromolecules could potentially weaken the strong electrostatic interactions holding the complex together, thereby aiding its separation.

Conclusions

In the doctoral thesis, I illustrated how, by incorporating the analysis of water molecules in biological systems such as protein, scientists can contribute to a much better understanding of macromolecule structure, dynamics, functioning and overall regulation. I showed that by analysing the behaviour of water molecules in proteins, we can contribute to such fields as drug design and protein engineering. I also confirmed that when studying water in enzymatic processes, we can reveal its multiple roles.

Conducting such comprehensive research would not be possible without the obtained knowledge about computational tools (**Paper 1**) that apply water molecules to the studies on macromolecules' properties. However, most importantly, it would be very hard to carry out the research without one software - AQUA-DUCT 1.0, in the development of which I was involved (**Paper 2**). In the field of drug design - I presented how, by using a combination of small-molecule tracking (for water and co-solvent molecules) and local-distribution approaches, it is possible to describe the variations in the dynamics of the internal pockets within the macromolecules and identify novel potential sites for ligand binding. By conducting such an analysis for the main protease of SARS-CoV-2, it was possible to indicate that targeting the active site binding pocket might not be the best strategy when designing inhibitors and that targeting other regions could be an alternative option (**Paper 3**). Additionally, the above-mentioned approach was also found useful in the assessment of the potential risk of off-target binding while studying it for SARS-CoV-2 Mpro and a panel of various proteases (**Paper 4**). Also, by using this approach, I was able to propose new potential binding sites for human soluble epoxide hydrolase (**Paper 5** and [12]). By conducting further *in silico* studies where these new sites were targeted, I was able to observe that there was a tendency for new potential inhibitors to bind strongly to these regions. During experimental validation, some of these compounds showed quite a strong inhibition effect, which gives hope that the predictions obtained using computational methods are correct. Regarding the protein engineering and the general protein regulation - I showed that, by tracking of water molecules during MD simulations, it is possible to describe in detail whole tunnel networks and the transportation phenomena in proteins. By using this approach, it was possible to determine the relationship between the structure and function in proteins from the soluble epoxide hydrolase subfamily (**Paper 6**). Additionally, it was finally possible to conduct a comprehensive comparison

of geometry-based and small-molecule tracking methods for detecting and analysing tunnels in proteins (**Paper 7**). By conducting an evolutionary analysis of the tunnels, a new theory was proposed on how tunnels can be formed in proteins (**Paper 8**). This might be very useful, especially in research aimed at carrying out the rational engineering of proteins, e.g. by *de novo* tunnel opening. Finally, as for the enzymatic reaction - I showed that water molecules can play various roles during the entire reaction cycle. Taking the example of the analysed proteolytic cleavage reaction in TLR8, even though the primary role of water (at a certain step of the reaction) is its catalytic role; it does not mean that water would not also have other functions. Based on the results, several additional roles of water molecules were hypothesised, e.g. provide the stabilisation for certain intermolecular interactions, act as a potential mediator in shuttling the proton between the specific amino acids or participate in the dissociation process of the protein-protein complex (**Paper 9, Preprint 1**).

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