Summary of Professional Accomplishments

1. Name

   Łukasz Pepłowski

2. Diplomas, degrees conferred in specific areas of science or arts, including the name of the institution which conferred the degree, year of degree conferment, title of the PhD dissertation

   - 2009 PhD degree in physics
     Faculty of Physics, Astronomy and Informatics;
     Nicolaus Copernicus University in Toruń (NCU).
     Doctoral thesis "Molecular dynamics and bioinformatics studies of enzymatic reaction mechanisms, with the focus on nitrile hydratase".
     Supervisor: prof. dr. hab. Wiesław Nowak
   - 2004 Master's degree in physics, specialization in computer physics,
     Faculty of Physics, Astronomy and Applied Informatics, UMK
     Master's thesis "Computer modeling of enzymatic activity of nitrile hydratase - an enzyme important in biotechnology".
     Supervisor: prof. dr. hab. Wiesław Nowak

3. Information on employment in research institutes or faculties/departments or school of arts

   - 2009-now: Assistant Professor, Faculty of Physics, Astronomy and Informatics;
     Nicolaus Copernicus University in Toruń.
   - II-III 2011: Researcher, R&D Department, Adamed Sp. z o.o. Pieńków.
   - 2008-2009: Assistant, Faculty of Physics, Astronomy and Informatics;
     Nicolaus Copernicus University in Torun.

4. Description of the achievements, set out in art. 219 para 1 point 2 of the Act.

   4.1. **Title of the achievement.**

   Application of theoretical computational biophysics methods for improving biotechnological enzymes.

4.2. Research objectives, results and description of publications based on them, impact on future research.

The scientific achievement that is the basis of this proposal is the application of theoretical computational biophysics methods for improving selected properties of biotechnological enzymes, with a particular focus on nitrile hydratase (NHase) [1], and sucrose phosphorylase enzyme (SPase) [2]. Both of these proteins are used on a mass scale in biotechnology companies, resulting in cheap and environmentally friendly production of a range of desirable chemical compounds. Both the scientific community and the biotechnology industry are looking for new tools to design improved enzyme variants. Genetic engineering makes possible the production of proteins that exhibit superior properties compared to natural-occurring enzymes (this self-presentation will often use the abbreviation WT from "Wild Type" to designate naturally occurring, otherwise native, proteins). Improved enzymes are also cognitively important. Their study provides insight into the molecular mechanisms that govern living matter, resulting in a better understanding of the world around us. For commercial applications, an improved enzyme saves the time and energy needed to produce many commercially essential compounds. We are observing a strong trend stimulated, for example, by ecology to use biotechnological processes for production, which are often much cleaner than classical chemical methods. A company that uses more efficient enzymes will have tangible financial benefits. Therefore, the need for advanced scientific research on enzymes, including biophysical research, is very high.

One of the possible approaches to improve enzymes may be the use of theoretical computational biophysics methods, more precisely, the methods of molecular dynamics and molecular docking. These methods, as shown, for example, in our publications, make it possible, with the support of bioinformatics and experimental biotechnology tools, to design new and improved enzyme variants relatively cheaply and in a short period of time Enhancement can mean, among other things, increased catalytic activity, increased thermostability or better selectivity towards selected substrates. Moreover, using the tools of theoretical computational biophysics, we can explain why improved proteins work more efficiently.

4.2.1. Introduction and research motivation.

Several decades ago, scientists noticed that it was often cheaper and more environmentally friendly to produce many chemical compounds using the most efficient machinery: enzymes. Many scientists believe that production technology is going to undergo a revolution, replacing traditional agriculture with food created

through bacteria that have enzymes embedded in them, which will create food cheaply and, most importantly, without using excessive amounts of water, energy and without emitting any greenhouse gases [3-6]. Due to the fact that there is a great need to both learn how enzymes work, but also to improve them, I became interested in the subject of improving enzymes. I realized that my broad knowledge of physics and theoretical computational biophysics methods in studying biomolecules, particularly non-standard enzymes, could support the experimental groups' work. As it turned out, theoretical and computational support proved to be extremely important in the process of designing two enzymes.

Having previous experience with the enzyme nitrile hydratase, which catalyzes the hydration of toxic nitriles, which are often waste products of chemical processes, e.g., in latex production, into valuable amides that can be used as soil improvers, feed additives (vitamin PP) or intermediates for making polyacrylamide clothing [7], and which possible catalytic mechanisms I investigated in my PhD thesis, I decided to collaborate with a group that investigates these enzymes experimentally. Because there are no such groups in Poland, a productive cooperation was established with a foreign scientific group from the School of Biotechnology, Jiangnan University, Wuxi, China, led by Prof. Zhemin Zhou. Our joint approach to the semi-rational design of more efficient enzyme variants has resulted in 10 scientific articles, most of which were published in highly-ranked journals. Thanks to the fruitful collaboration with Prof. Zhou, several visits to China, I was able to expand my collaboration with Jiangnan University with Prof. Xianzhong Chen's group. Using a similar approach to NHase, the properties of the sucrose phosphorylase enzyme have been improved through computational calculations, resulting in two scientific papers published so far. Our innovative approach to semi-rational improvement of biotechnological enzymes using the methods of theoretical biophysics and experimental biotechnology is unique because only a few groups in the world apply such an approach to non-standard enzymes such as nitrile hydratase. NHase contains posttranslationally modified amino acids and a metal ion in the catalytic center, making inexperienced scientists without in-depth knowledge of theoretical computational biophysics methods unable to model and study the properties of such enzymes. It is worth mentioning here that Jiangnan University in the Shanghai 2022 ranking was ranked as 44th in the discipline of biotechnology and as first in food science and technology (world ranking).

One of the most widely used methods of theoretical computational biophysics is classical molecular dynamics (MD) [8]. Briefly, the MD method is based on numerical integration of Newton's equations of motion (4.1), which allows to simulate the evolution in time using classical approach, of a model describing a complex physical system built, for example, of atoms with masses m_i and positions r_i:

$$\vec{F}_i = m_i \frac{d^2\vec{r}_i}{dt^2} \quad (4.1)$$

In the case of modeling biological systems, this is realized with the consideration of numerous atom-atom interactions in order to obtain information about the properties of living matter in an environment that most closely approximates the natural environment of the studied system, such as an aqueous environment. The interactions above include covalent bonds, electrostatic interactions, van der Waals interactions or hydrogen bonds. These interactions cause all atoms interact with each other with forces determined by a specific analytical function that describes the potential energy and a set of parameters of this function. This function is commonly called the force field [9] (4.2).

$$\begin{aligned} V(r) = &\sum K_b(b-b_0)^2 + \sum K_\theta(\theta-\theta_0)^2 + \sum K_{UB}(s-s_0)^2 + \\ &\sum K_\chi(1+\cos(n\chi-\delta)) + \sum K_{imp}(\varphi-\varphi_0)^2 + \\ &\sum \varepsilon\left[\left(\frac{R_{\min_{ij}}}{r_{ij}}\right)^{12} - \left(\frac{R_{\min_{ij}}}{r_{ij}}\right)^{6}\right] + \sum \frac{q_i q_j}{\varepsilon_e r_{ij}}. \end{aligned} \quad (4.2)$$

The force field represents the potential energy of a static system of interacting atoms. Having the potential defined for the system, the forces acting on each atom can be determined (4.3):

$$\vec{F}_i = -\vec{\nabla}_{r_i} V(\vec{r}_1,...,\vec{r}_N). \quad (4.3)$$

With the forces acting on the model's atoms at a given time t determined in this way, the positions of the atoms in the next step, or future time, can be calculated (4.4):

$$\vec{r}(t+\Delta t) = \vec{r}(t) + \vec{v}(t)\Delta t + \frac{1}{2}\frac{\vec{F}(t)}{m}\Delta t^2. \quad (4.4)$$

To do this, information about the velocities of the atoms is still needed. The initial velocities of N atoms are usually generated randomly according to the Maxwell-Boltzmann distribution and with a given temperature T of the system, combining the kinetic energy of the atoms with the principle of energy equipartition (4.5):

$$\frac{1}{2}\sum_{i=1}^{N} m_i \left\langle v_i^2 \right\rangle = \frac{3}{2} N k_B T, \quad (4.5)$$

where k_B is Boltzmann's constant.

The velocities v of the atoms in the next steps are calculated according to equation (4.6):

$$\vec{v}(t+\Delta t) = \vec{v}(t) + \frac{\vec{F}(t) + \vec{F}(t+\Delta t)}{2m}\Delta t, \quad (4.6)$$

Equations (4.4) and (4.6) are called Verlet's velocity algorithm [10, 11]. The CHARMM 27 force field was used consistently in all the studies presented as the habilitation achievement [9, 12]. To describe the interactions of the non-standard catalytic center of nitrile hydratase, parameters and topologies compatible with the CHARMM force field, developed based on quantum calculations completed during the PhD studies, and used for the first time in a paper regarding the transport of NHase ligands inside the protein [13] (purely theoretical work), were used.

The method of computer aided docking of small ligands to proteins involves an algorithmic search for such a mutual placement of the ligand and protein that the potential energy function, e.g. in the form (4.2), takes on the minimum possible value [14, 15]. I used docking via the Lamarckian genetic algorithm implemented in AutoDock 4.2 [16]. Unfortunately, none of the numerous commercial programs tested allowed docking to NHase containing a non-standard center with a Co3+ cobalt ion. AutoDock was the only tool that allowed the docking of ligands to NHase. By modifying standard computational protocols and with the ability to introduce custom parameters describing the atoms, AutoDock allowed to predict realistic positions of ligands inside the protein, which I published while I was still working on my PhD dissertation [17], and which was later confirmed experimentally [18].

With computational biophysics methods combined with bioinformatics tools, I studied mainly the enzyme nitrile hydratase (NHase, EC 4.2.1.84) [1, 19]. This enzyme catalyzes the hydration of toxic nitriles to very useful amides (Fig. 1).

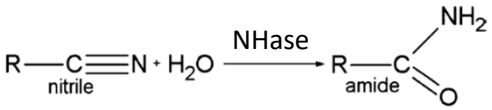

Fig. 1. Scheme of the reaction carried out by nitrile hydratase.

The smallest active NHase subunit is an αββα heterotetramer composed of two identical αβ heterodimers (Fig. 2a). Each dimer contains a non-standard active site containing a cobalt (Co^{3+}) or iron (Fe^{3+}) ion, two posttranslationally modified cysteines (sulfinic-cysteine (CSD) and sulfenic-cysteine (CEA)), and a standard cysteine and serine (Fig. 2b).

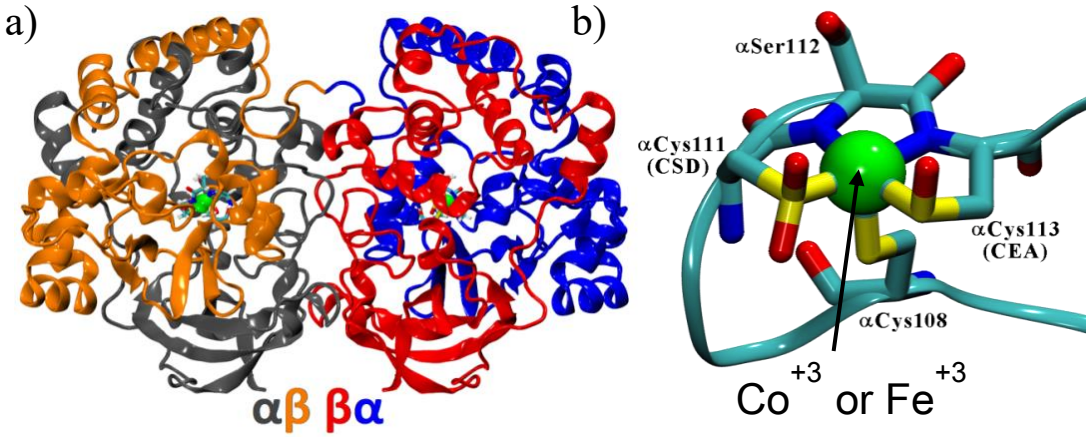

Fig. 2. Nitrile hydratase heterotetramer with specified chains and marked active site (a). Non-standard NHase active site with marked ion metal (b). In panel (b), the amino acid numbering follows the crystallographic structure 1IRE.

NHases with different amino acid sequences are quite a lot. According to the RefSeq database [20] as of 1/10/2022, 4823 alpha subunit sequences and 5432 beta subunit sequences were known. Often, the differences in amino acid composition can be significant and the number of identical amino acids can be less than 40% [21] Nitrile hydratase, depending on the organism from which it is derived, can exhibit different catalytic activities towards given substrates, e.g. NHase from the bacterium *Rhodococcus sp*. N-774 shows activity in the forming of acrylamide of 500 g/(g cells) and *Rhodococcus rhodochrous* J1 of 7000 g/(g cells) [22, 23]. Increasing the catalytic efficiency of this enzyme is essential from the point of view of biotech companies, as they can produce much more amides in the same time.

The thermostability of NHases can vary, as well. NHase from *Pseudomonas chlororaphils* B23 [24] and *Rhodococcus sp*. N-774 are stable at temperatures below 20ºC [25], while NHase from *Rhodococcus rhodochrous* J1 is stable only between 10ºC and 30ºC [26]. A variant of NHase from the thermostable organism

Pseudonocardia thermophila JCM3095 is stable even at 50ºC, although after just 18 minutes it shows only half of its initial activity [H4]. Thermostability is one of the key factors in the practical use of NHase in the industry. The hydration reaction of nitriles is an exoenergetic reaction [19]. Therefore, the bioreactors in which the reaction is carried out heat up strongly. They can be cooled (which is uneconomical), or an engineered enzyme that is more stable at high temperatures can be used. Besides, the bioreactor's increased temperature prevents unwanted microorganisms' growth [27]. Therefore, new, more thermostable NHases have either been searched for or designed for many years.

Another key factor for enzymes is their selectivity [28, 29]. Many enzymes exhibit selectivity to only one substrate. In contrast, other enzymes can catalyze a whole group of compounds and perform the same type of reaction of many different substrates containing a given functional group. In the rational design of the selectivity of enzymes, we most often want to force the enzyme to catalyze from a mixture of similar compounds one selected substrate or to broaden the spectrum of catalyzed compounds. The product of catalysis can become either an effective, pure, and inexpensive drug or its intermediate [30, 31]. As a rule, nitrile hydratases exhibit a broad catalytic spectrum, although variants of NHases showing selectivity to one of the optical isomers are known [32-34]. However, mostly efficient NHases used on a mass scale in the industry do not show particularly significant selectivity, while pharmaceutical, as well as chemical industries, are very interested in obtaining various compounds (e.g., drugs or their intermediates) derived from pure biosynthesis [19, 31].

The success of nitrile hydratase in the biotechnological conversion of nitriles to amides is evidenced by the scale of its application. Publications report that more than 600,000 tons of acrylamide are produced annually using NHase [35]. These numbers (dating back to 2015) are severely underestimated. As a result of Professor Zhou’s group’s collaboration with two, of at least eleven companies located in China (Jiangsu Changjiu Agrochemical Co., Ltd and Xinyong Biochemical), I know that at the end of 2019 they each produced at least 50,000 tons of acrylamide per year. In addition to the above-mentioned Chinese biotech companies, acrylamide, nicotinamide (vitamin PP), 5-cyanovaleramide (an intermediate of herbicides and epilepsy drugs), using NHase are also produced in Japan, Korea, Germany, France or the United States [19, 24, 36].

4.2.2. Results and description of publications based on them.

The results of the studies on the design of NHases with improved properties mentioned above (i.e., catalytic activity, thermostability, and selectivity) and the explanation of the reasons for the improvement of the above-mentioned properties are presented as the main scientific achievements of the habilitant. It seems that this task is a biochemical or biotechnological problem, but decent modeling requires advanced methods of theoretical molecular biophysics. Only an interdisciplinary approach, with strong participation of physical methods, has given important achievements in designing enzymes with highly improved parameters.

In scientific publications [H1], and [H2], I focused on explaining the reasons for the enhanced activity of NHases. The papers [H3] and [H4] also reported the development of enzymes with enhanced activity, however, the main focus of these papers was an explanation of how to design a more thermostable variant of the enzyme using the tools of theoretical biophysics and bioinformatics as well as a detailed explanation of the reasons for the improved thermostability. In the paper [H5], I explained using molecular modeling techniques why some variants of NHases with additional protein fragments (so-called linkers) show greater thermostability than native proteins. To show that using the tools of theoretical biophysics, the thermostability of other biotechnology enzymes can be increased, in the paper [H6] an almost identical approach to the paper [H4] was applied but to the enzyme sucrose phosphorylase (SPase). The papers [H7] and [H8] show how, using molecular modeling, design enzyme variants that exhibit stereoselectivity or catalyze only one nitrile group in symmetric dinitriles and then explain their new catalytic properties.

Studies on enhancing the catalytic activity of enzymes.

As mentioned above, the smallest NHase unit exhibiting catalytic activity is the αββα tetramer. Physicists using models often try to simplify them so that, despite the simpler description, reliable research data can still be obtained. Until a few years ago, access to computing power was so limited that MD simulation times of biological systems were single tens of nanoseconds. The approach used for proteins composed of several identical subunits was to study a single fragment and extrapolate the results to a larger system. A similar approach was used for nitrile hydratase, where initially only a single αβ dimer was studied using theoretical biophysics techniques. In fact, all NHase structures available in the Protein Data Bank [37] (PDB, a data bank of spatial

structures of biomolecules) are precisely dimers, and tetramers are obtained only by appropriate symmetry transformations. In the research teams and publications that I know of, no one but me uses the full NHase tetramer model in theoretical studies. It is common to use a simplified dimer model. No one besides me also uses a full and realistic active site model in MD simulations. Other groups use an apoenzyme without a cobalt (iron) ion and without posttranslational modifications in their studies [38-41], and therefore do not include all interactions in their models. The reason for this is the impossibility of generating parameters and topologies [42, 43] for the active site embedded in the protein. The parameters and topologies developed by me in agreement with the CHARMM 27 force field on the basis of quantum-chemical calculations carried out as part of my doctoral dissertation make it possible to create a more accurate, and therefore more realistic, physical model.

Molecular dynamics simulations can be used successfully to explain the causes of increased enzyme activity. A good example is the study of NHase from the thermophilic bacterium *Pseudonocardia Thermophila* JCM 3095 (*Pt*NHase). It has been known for many years [44], and in 2001 its crystallographic structure was solved and placed in the Protein Data Bank (pdb code 1IRE) [45]. Despite its origin in a thermophilic organism and its application in biotechnological conversion of nitriles to amides, this enzyme has one major drawback - low activity against aromatic nitriles in particular, e.g., nicotinonitrile or benzonitrile. I started research on a variant showing improved catalytic activity together with the Chinese group in 2018. At that time, we studied the βM46K variant of *Pt*NHase, which showed a 3.8-fold increase in catalytic activity against benzonitrile (176 U/mg in the WT, 668 U/mg in the βM46K variant). MD simulations showed that replacing a hydrophobic amino acid (methionine) with a hydrophilic amino acid (lysine) it changes orientation strongly. The side group of the hydrophilic amino acid being initially burried inside the protein in all the simulations changed orientation moving out into the solvent. In the case of protein simulations, this is water (TIP3P model). This resulted in the widening of the pocket located above the active site. The subsequent crystallographic structure confirmed the predictions of the MD simulations (Fig. 3).

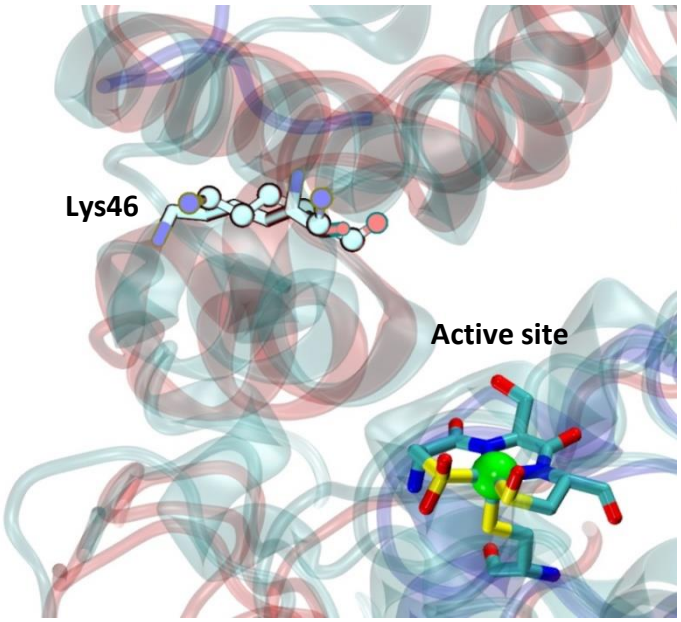

Fig. 3. Crystallographic structure of NHase βM46K (cyan color) with marked lysine 46 (balls and sticks representation) and superimposed structure obtained after 20 ns of molecular dynamics simulation (red color) with marked lysine 46 (sticks representation).

These data (except for a master's thesis by Ms. Lan Jao of the School of Biotechnology, Jiangnan University, CHINA) have never been published.

Tunneling analysis of the WT 1IRE protein, which I performed using the CAVER tool [46] showed that βMet46 together with the amino acids βPhe41, βGly47, βLeu48, βLeu127, βPro128, βAla129, βArg131, βIle177 and βGlu188 create the initial part of the channel leading to the active site. Analysis of this enzyme using the ConSurf server [47] showed that all amino acids building the entrance to the channel except βPhe41, βMet46, βLeu127, βPro128, and βAla129 are highly conserved and should not be modified. The Chinese team performed saturated mutagenesis tests on the non-conserved amino acids. Analysis of all 100 variants showed that the two mutants βM46R and βA129R of *Pt*NHase exhibit particularly high catalytic activity against many nitriles. We, therefore, decided to investigate the reasons for this positive catalytic activity by comparing the data with the native protein. We published the results of our study in the paper [H1].

Table 1 shows the catalytic activities of the native hydratase and the two most efficient variants βM46R and βA129R (the publication also shows the activities of other variants). Interestingly, in the case of the very large nitrile tyocamycin, the native variant originally had no activity, while both mutational variants were able to carry out hydration of this nitrile to give sangivamycin, a nucleotide analogue that is an antibiotic, antiviral and anticancer drug [48, 49].

Table 1. Activity of native *Pt*NHase and its mutational variants against selected nitriles. Source [H1].

	Specific activity (U mg^{-1})				
Substrate	WT	M46R	Fold	A129R	Fold
Acrylonitrile	2463 ± 153.8	3056 ± 251.3	1.2	2542 ± 236.8	1
Isobutyronitrile	666.2 ± 80.8	3720.5 ± 77.3	5.6	1498.9 ± 89.6	2.3
Pentanenitrile	14.4 ± 0.9	118.4 ± 3.7	8.2	60.5 ± 15.3	4.2
Hexanenitrile	1982.4 ± 83.8	5613.3 ± 112.9	2.8	1503.6 ± 154.8	0.8
2-Cyanopyrazine	203.2 ± 8.1	851.2 ± 8.1	4.2	660.4 ± 4.0	3.3
3-Cyanopyridine	66.7 ± 1.0	396.4 ± 8.3	5.9	661.8 ± 69.0	9.9
Benzonitrile	176.5 ± 8.1	1704.7 ± 11.2	9.7	1211.9 ± 17.6	6.9
Cinnamonitrile	45.8 ± 1.0	282.9 ± 12.3	6.2	379.1 ± 5.2	8.3
1-Naphthonitrile	13.1 ± 1.7	84.3 ± 4.2	6.4	20.6 ± 1.8	1.6
Thiacloprid	5.4 ± 0.3	42.2 ± 1.0	7.8	78.9 ± 2.3	14.6
Toyocamycin	0	12.6 ± 1.2[a]	—	4.2 ± 1.2[a]	—

The reason for the improved activity probably lies in the changed structure of the mutants relative to WT. To find out this mechanism of improved catalysis, crystals of these proteins were obtained jointly with the Chinese side and the spatial structure of both variants was solved. The crystallographic structures were deposited in the Protein Data Bank with the codes 7W8L - βM46R variant and 7W8M - βA129R variant. My task, in this case, was to validate the crystallographic data (e.g., to detect deficiencies in amino acid matching, especially posttranslational modifications and the proper structure of the active site) as well as to compare the new structures to the native 1IRE structure. The analysis led to interesting results. I noticed that, as in the case of *Pt*NHase βM46K, in the βM46R variant the arginine is oriented towards the solvent, changing the arrangement of the amino acids composing the catalytic pocket. Arginine in the β-chain at position 129, however, forms a salt bridge with βAsp49, making the arginine side chain partially oriented toward the solvent. The native *Pt*NHase hydrophobic alanine side group had the alanine side group oriented toward the inside of the protein (buried). Unfortunately, the static structures did not provide a complete answer as to why the new enzyme variants showed better catalytic activity than WT. Crystalographic structures analysis with the CAVER tool showed that the entrance to the catalytic center is slightly larger in the modified proteins, but just beyond this area is a narrow passage - a very similar crossing in all cases (Fig. 4. in publication [H1]). MD simulations gave me a complete picture of the reasons for more efficient catalysis in the PtNHase variants. For each variant, I calculated 200 ns trajectories at 300K.

Analysis of RMSD plots (from Root Mean Square Distance; Fig. S7 in [H1]) showed that globally all *Pt*NHase variants behaved correctly (stable) during simulations and similarly to simulations performed for other NHases. Fluctuation analysis (otherwise known as RMSF, from Root Mean Square Fluctuations, Fig. 4a

and 4b) highlighted large differences in the β-chain in the amino acid 181-187 region (Fig. 4b). In the native protein, I noticed much higher amino acid mobility in this area than in both mutational variants. Analysis of amino acid contacts (a contact in this case is considered to be when any atoms from two amino acids approach each other at a distance of less than 4 Å) at positions 46 and 129 in the β-chain showed that the native amino acids interact differently than the mutant ones (Fig. 4c and 4d). In the case of the amino acid βArg46, contacts with amino acids forming the catalytic pocket walls (e.g., with βPhe51, βPhe41, βTyr76) disappear completely and new interactions appear (e.g., with βThr83, βArg82).

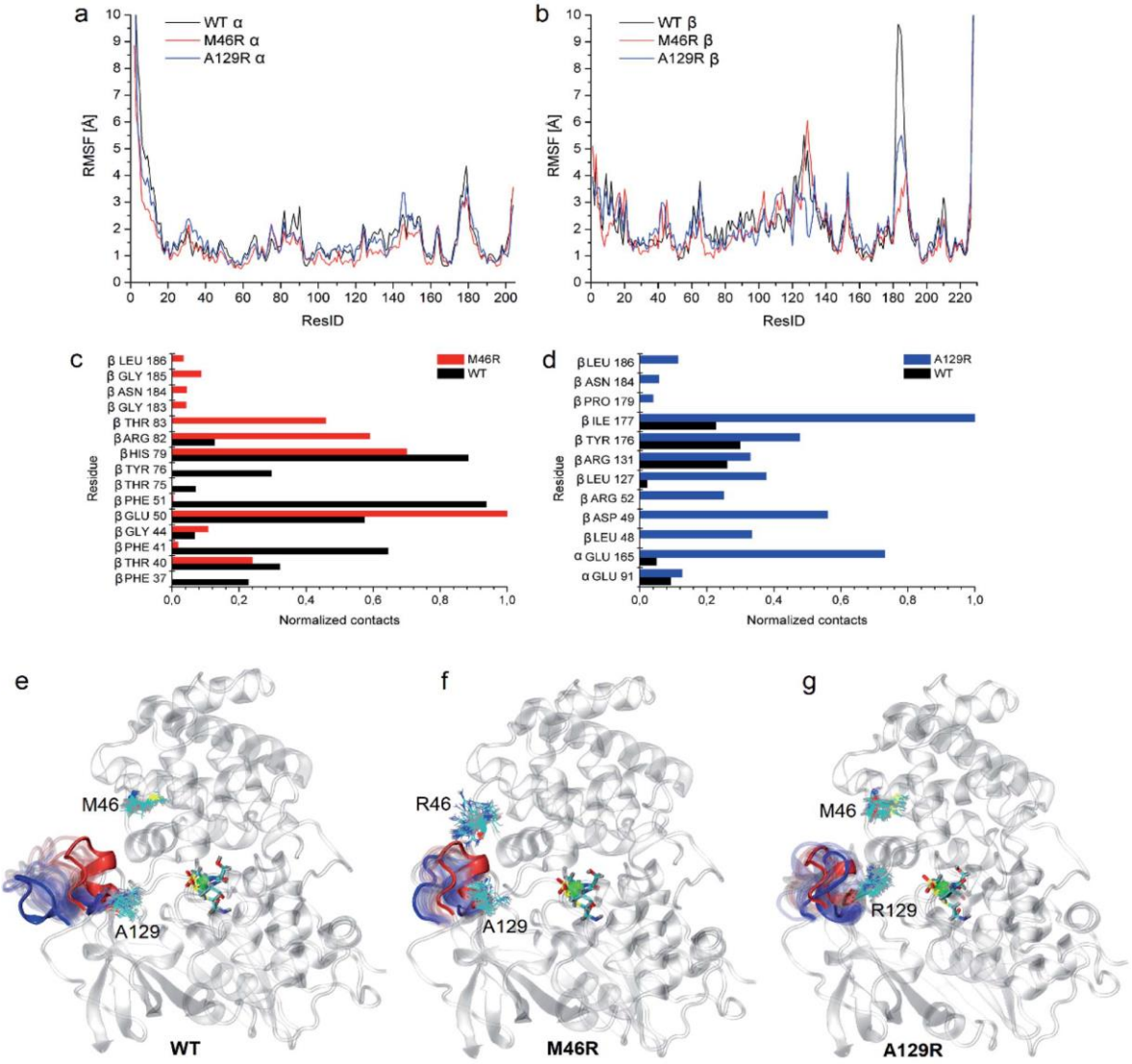

Fig. 4. Molecular dynamics simulation trajectory analysis. Panels a and b show plots of α and β chain fluctuations. Panels c and d compare amino acid contacts at positions 46 and 129. Panels e, f and g show the effect of mutations on amino acids β181-189. Source [H1].

At position 129, alanine in WT has a small number of contacts with the amino acids βArg131, βTyr176, βIle177 (Fig. 4c). The mutation introduced with arginine results in an increase in the number of contacts with the above amino acids, and in addition, new contacts appear with, for example, αGlu165, βAsp49 (salt bridge), βArg52, βLeu127 and to a minor extent with amino acids 179-186 (Fig. 4d). The newly formed interactions stabilize amino acids 181-187 of the β chain (Fig. 4e, 4f, 4g). This area is close to the entrance of the catalytic center. Additionally, thanks to mutagenesis, new interactions were made with the amino acids necessary for catalysis (βArg52) [50], stabilizing the correct structure of the active site.

Both mutations result in an enlargement of the pocket above the catalytic center. My analysis of the MD simulation results using the CAVER program showed that in the native protein the pocket had a volume of 178.2 ± 55.0 $Å^3$, while in the modified enzymes they are 193.7 ± 40.4 $Å^3$ and 206.9 ± 61.3 $Å^3$, which means that larger substrates such as tyocamycin can approach the metal ion and can be hydrated.

Another factor that increases catalytic activity toward large amino acids is a change in the cross-correlation of the motions of large fragments of the β-subunit, i.e., amino acids 37 to 97 with amino acids in the 107-227 range. In the native protein, the movement is weakly correlated, the introduction of the mutation causes an anti-correlated movement which makes the β-subunit perform low-frequency breathing oscillations (so-called breathing mode), and consequently this gives a more frequent opening of the channel leading to/from the active site (Fig. 5), and as a result increased catalytic activity.

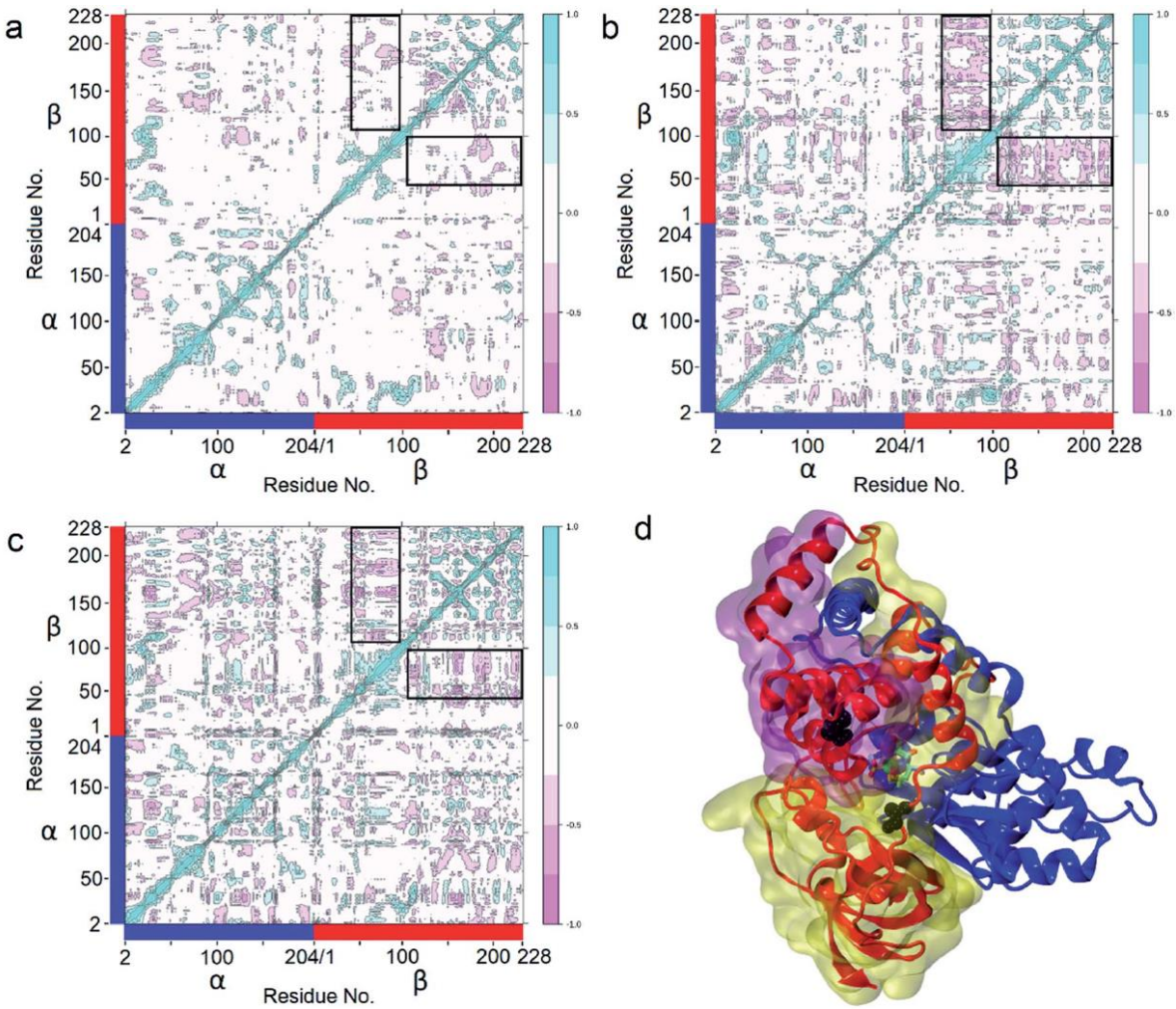

Fig. 5. Cross-correlation matrices calculated from MD simulations for native protein (a), *Pt*NHase M46R (b), and *Pt*NHase A129R (c). In panel (d), regions of the β-chain showing cross-correlation of low-frequency motions are marked. Amino acids β37-97 are marked in purple and β107-229 in yellow. Source [H1].

The next issue in elucidating the enhanced catalytic activity was the study of a thermostable nitrile hydratase from the bacterium Streptomyces thermoautotrophicus (*St*NHase) [H2]. A major issue, in this case, was the lack of crystallographic structure of *St*NHase. The initial positions of the atoms are essential for the realization of MD simulations, so a PhD student, Julia Berdychowska, MSc (I am her associate supervisor), developed a homology model of the tetramer under my supervision using the SwissModel tool [51] (Fig. S5 in the supplement [H2]). According to the evaluations of 3D structure verification programs [52-54], this model has very good quality. In addition, this was confirmed by the elemental analysis of MD simulations, in which the values of RMSD (Fig. S6 in the [H2] supplement) and RMSF (Fig. S7 in the [H2] supplement) were very similar to those from simulations previously performed by us and by other research groups [H1], [H4], [H5], [41]. Similarly to

[H1], in this problem I also used the CAVER tool [46] to identify the amino acids that form the entrance to the catalytic center. These were the amino acids βLeu37, βPhe41, βTyr46, βLeu48 and βPhe51. All of the above amino acids were mutated by the Wuxi collaborators into proline, lysine aspartic acid, alanine, phenylalanine and arginine, respectively. Experiments showed that the variant showing the best catalytic activity was the βL48D mutant, with catalytic activity against picolinonitrile increasing 3.7-fold (from 65.44 U/mg to 242.75 U/mg) and against the chemically important nicotinonitrile up to 7.7-fold (from 73.8 U/mg to 566.18 U/mg) relative to WT. I examined the native and mutational variants together with the above mentioned PhD student using theoretical computational biophysics methods.

We prepared 200 ns trajectories at 300K. We jointly analyzed the results, which showed that, despite the use of the homology model, the values of RMSD and RMSF (Fig. S6 and S7 in supplement [H2]) are similar to those obtained in other works, which means that globally the model behaves similarly to the crystallographic structure. We carefully studied the behavior of amino acid No. 48 (Phe/Arg) of the β chain in the native protein and the mutational variant. It turned out that the large and hydrophobic leucine splits the channel leading to the active site (Fig. 6a) into two parts, while the hydrophilic and much smaller aspartic acid does not partition the channel (Fig. 6b). I estimated the volumes of both channels. The one in the native protein is much smaller, with a volume of 143.5±52.7 $Å^3$, while the one in the L48D

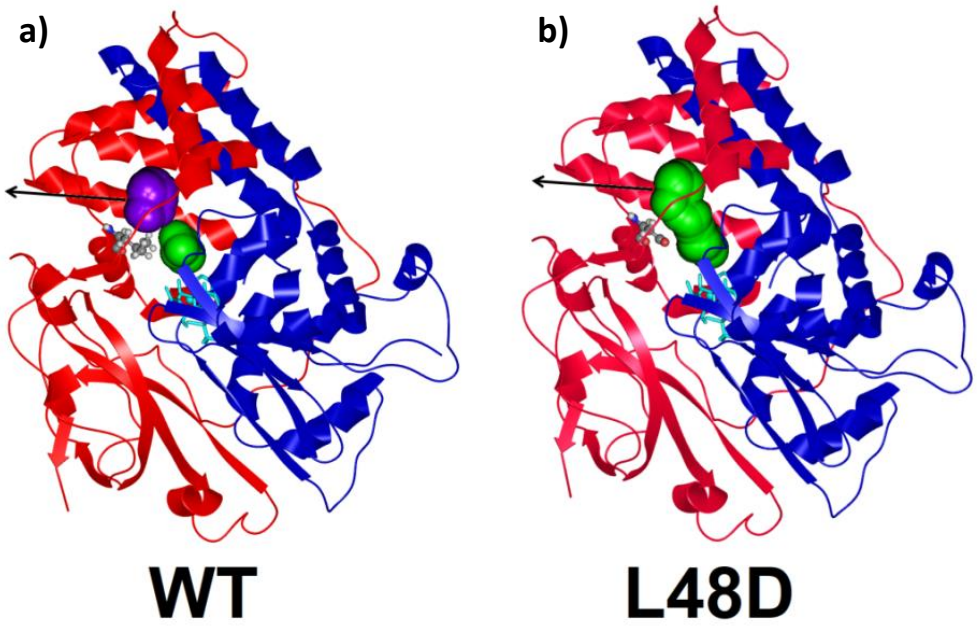

Fig. 6. Channels leading to the active site of *St*NHase in the native variant marked in purple and green (a) and in the L48D variant marked in green (b). The channel was determined by analyzing 11 simulation frames each obtained every 20 ns, merging information from both dimers. Source [H2].

variant has a volume as large as 285.1 ± 133.4 Å^3. This fact explains why large substrates can more easily enter the catalytic center in the mutant.

Detailed analysis of the MD trajectory of leucine/aspartic acid showed that leucine mainly takes one position in which the dihedral angle between the C-Cα-Cβ-Cγ atoms is about 60° and rarely takes an angle of about 170° (Fig. 7a, I called the C-Cα-Cβ-Cγ angles of 60° as conformation A, while those taking a value of 170° as conformation B). Taking the C-Cα-Cβ-Cγ atoms of leucine an angle of about 170° allows the formation of a large passage between the two parts of the channel. Aspartic acid, which prefers to interact with the solvent, very rarely adopts conformation A (from Fig. 7), while it very often flips towards the solvent being in conformation B or C (shown in Fig. 7). This causes, firstly, an enlargement of the channel leading to the catalytic center (conformation B) or even its opening (conformation C) creating, according to the terminology of the CAVER program, a pocket (i.e. a channel with direct access to the solvent). Analysis with the CAVER program showed that in about 30% of the analyzed structures obtained from simulations of the L48D variant, the tunnel is open, while in the native protein the channel leading to the catalytic center never fully opens.

I also noticed that aspartic acid in the C-conformation stabilizes the very important Arg52 for catalytic activity [50], which also probably has an impact on the enhanced catalytic activity of NHase.

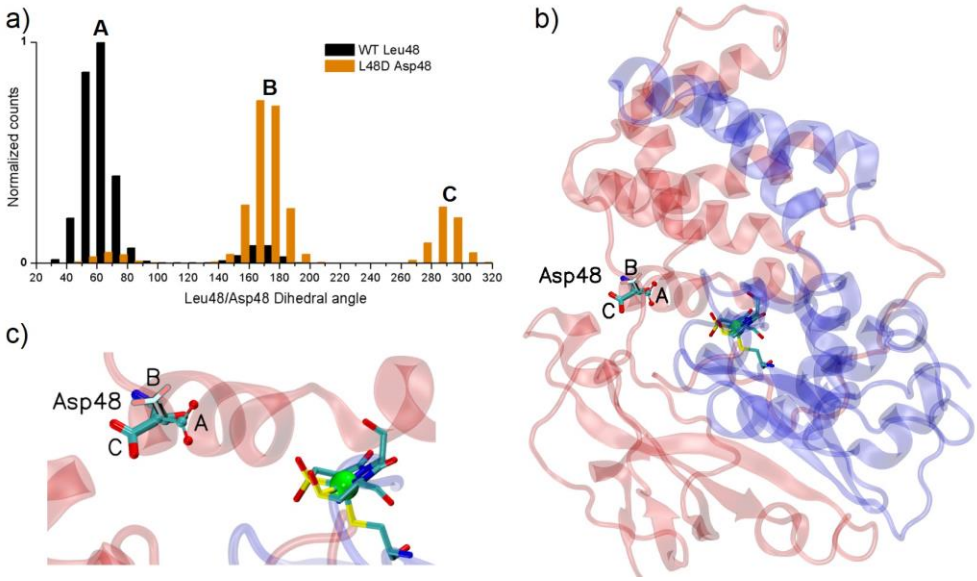

Fig. 7. Dynamics of leucine 48 in native *St*NHase and aspartic acid in the L48D variant of *St*NHase. Panel (a) shows histograms of the dihedral angle values between the atoms of the C-Cα-Cβ-Cγ for both NHase variants. Panels (a) and (b) show aspartic acid alignments representing individual maxima read from the histogram. Source [H2].

The above two examples of studies using theoretical computational biophysics methods show that a molecular dynamics approach in which we use a classical strategy to determine the trajectories over time of a large number (about 75,000) of interacting atoms can capture the effect of changing just a few atoms on the behavior of such a large biomolecular system. Using MD methods in combination with bioinformatic analysis and then experimental biotechnology methods, it is possible to design more efficient enzymes and, equally important, explain why they work better. Without the use of theoretical biophysics methods, designing more efficient enzymes alone usually takes much longer and often resembles roulette, where better variants are obtained randomly, while rationally explaining exactly why an enzyme works better with the current experimental methods is practically impossible.

Improving the thermostability of biotechnological enzymes.

Improving thermostability is another important factor in designing better enzymes. There are several reasons for this. Typically, chemical reactions take place faster at higher temperatures. In addition, the increased temperature of the bioreactor results in much slower growth of unwanted microorganisms such as fungi and bacteria that negatively affect, for example, the purity of the compounds obtained [55-57]. In the case of nitrile hydratase, it is important because the performed nitrile hydration reaction is exoenergetic. This causes a significant temperature increase in the bioreactor; therefore, it must be cooled, raising the cost of production [19, 58]. Consequently, a lot of studies are devoted to increasing the thermostability of enzymes.

Three publications ([H3], [H4], [H5]) showing how the thermostability of various nitrile hydratases can be improved are included in this achievement. To show that the methods are universal, in the article [H6] we proved that they also work for a completely different class of enzymes: sucrose phosphorylase, which products are used in the food and cosmetic industries.

My first article about increasing the thermostability of nitrile hydratase was a publication [H3]. It investigated the possibility of increasing the thermostability of NHase from the bacteria *Pseudomonas putida* NRRL-18668 (*Pp*NHase). *Pp*NHase is a highly efficient enzyme with insufficient thermostability to be used in industrial amide production. Previous studies without my participation have shown that simply linking two αβ subunits by a short linker (Pro-Gly, named in the publication [H3] Fus-

NHase) increases the thermostability of the enzyme [59]. Half of the enzyme's initial activity is obtained during a time 2.8 longer than the native variant (about 30 min), which is still insufficient for industry. I applied a semi-rational design using two computational biophysics tools to improve thermostability. The first was the ROSETTA 3.4 program [60] used, for example, to design more stable enzyme variants. The second tool was MD simulations of the enzyme. Since the ROSETTA program requires the 3D structure of the protein, I built a homology model of Fus-NHase, using the crystallographic structure of *Pp*NHase with the code 3QXE as a model [61]. Using potential energy minimization, I predicted the spatial structure of the loop connecting the two subunits (Fig. 2b in [H3]). ROSETTA identified in total 234 single mutations that could have an impact on thermostability. To be able to design a more thermostable Fus-NHase in a reasonable time and cost, I used the MD method to determine the regions of the NHase with the lowest stability, where the mutations proposed by ROSETTA could be introduced.

Analysis of the MD Fus-NHase results (carried out for the equivalent αβ dimer, i.e., for one linked chain), and in particular the fluctuations (Fig. 3a in [H3]), showed high lability of the protein in the range corresponding to the amino acids of the β chain from number 100 to number 219. Prof. Zhou's team experimentally examined all the mutations I proposed (using the ROSETTA program) in this area. There were 17 of them, 10 of which showed increased thermostability by at least 10% and four variants additionally showed increased activity against nicotinonitrile (Table 1 in [H3]). The top three variants βM150C, βT173Y and βS189E showed activity increased by 3.5%, 49% and 22% and thermostability calculated as the time at which the enzyme exhibits half of its initial activity at 50°C increased from 26 min to 40 min, 32 min, and 60 min, respectively, I tested using MD simulations. The calculations showed that all mutational variants exhibited better stability, as seen in the RMSD and RMSF plots (Fig. 5 in [H3]). A closer analysis of the MD trajectories showed that in all variants, thanks to good mutations, it was possible to increase the number of hydrogen bonds, especially those lasting for most of the simulation time, which stabilized the whole protein. It is worth noting that more thermally stable proteins have often increased catalytic activity as well [55, 57] (but this is not the rule).

A more sophisticated approach to improving enzyme thermostability was used in a study published in the paper [H4]. The FireProt [62] server analyzing *Pt*NHase (PDB

code 1IRE, from the paper [H1]) determined 10 mutations: αI5P, αT18Y, αQ31L, αD92H, βA20P, βP38L, βF118W, βS130Y, βC189N, and βC218V. The introduction of these mutations was expected to increase the thermostability of the enzyme: (Fig. 8). The variant containing all of the above mutations, according to [H4], was named M10. Initially, to test the prediction efficiency of the FireProt server, a series of short 30 ns simulations of αβ dimers of the native protein and M10 were performed (unpublished data). The promising results led my collaborators in China to perform an experimental study. It turned out that thermostability increased strongly, the native variant at 50°C showed half of its initial catalytic activity after only 18 min, while the M10 variant obtained 50% of its initial activity only after 120 min (Fig. 3 in [H4]). Moreover, the initial activity toward nicotinonitrile of the M10 variant was more than twice that of the native protein (168.8 ± 5.3 U/mg and 81.1 ± 2.8 U/mg, respectively).

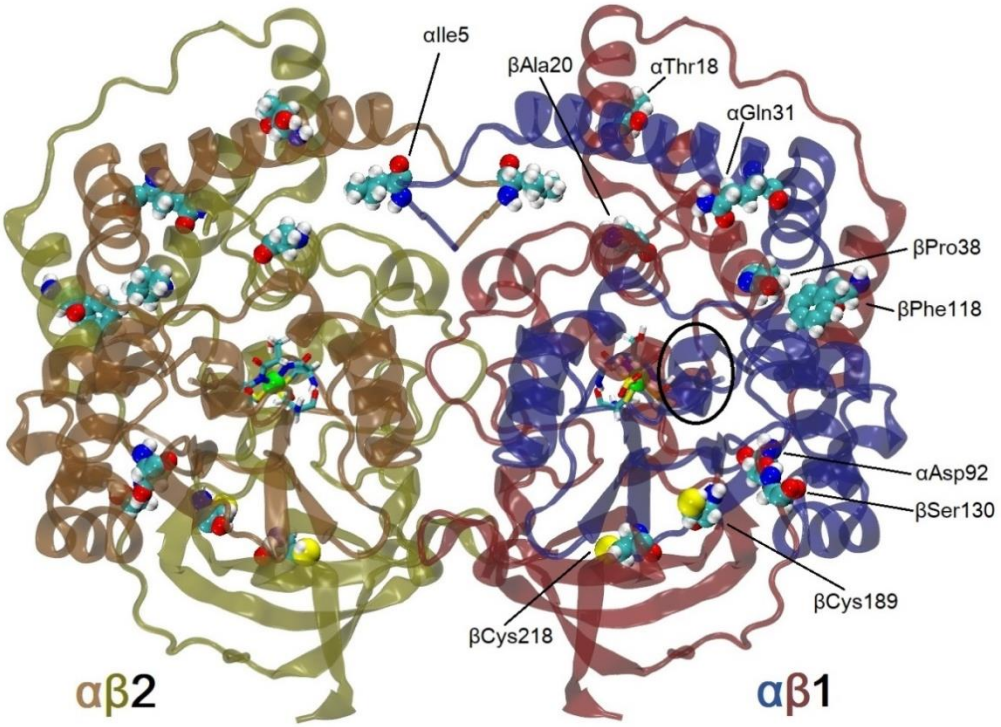

Fig. 8. Tetramer of native *Pt*NHase used in molecular dynamics with mutation sites marked. Source [H4].

Trying to explain the reason for the increased thermostability, I performed MD simulations of the dimers at two different temperatures: standard 300K and increased to 335K. Historically, these were the first such long MD simulations of NHase. Unfortunately, usually after about 50 ns the protein started to unfold. In order to improve the stability of the system, I created a tetramer model for the first time based on "Biological Assembly" - additional 3D structure data deposited in the Protein Data Bank together with the 1IRE structure of *Pt*NHase. It turned out that the αββα tetramer

models in simulations remain more stable than αβ dimers (unpublished data, we are currently running simulations on the order of 500 ns and have no problem with the stability of the tested models; between αβ dimers there are mainly so-called "hydrophobic interactions"). The publication [H4] was the first paper in the world literature presenting the results of MD simulations of the NHase tetramer.

Using MD simulations, I explained the global impact of the introduced mutations on the enzyme's dynamics. Using Principal Component Analysis (PCA), I identified those fragments of the protein that undergo the greatest stabilization after the introduction of mutations (Fig. 7-8 in [H4]). By stabilizing the entire protein in the M10 variant, the structure of the active site and the surrounding Arg52 and Arg157 important for catalytic processes was much better preserved (Fig. 9 in [H4]). Through analyzing the enzyme radius of gyration, I showed that the modified enzyme becomes more compact, which is beneficial for increasing protein thermostability [63].

Through the MD simulations I performed, I showed at the molecular level how each of the 10 mutations influence the enhancement of protein thermostability. For example, changing an isoleucine at position 5 to a proline in the α-chain (αI5P) results in a stiffening of the loop from positions 4 to 7 in the α-chain, and this results in the production of a salt bridge between αGlu3 and αLys8 (Fig. 9). Similar loop stiffening is caused by the βA20P mutation.

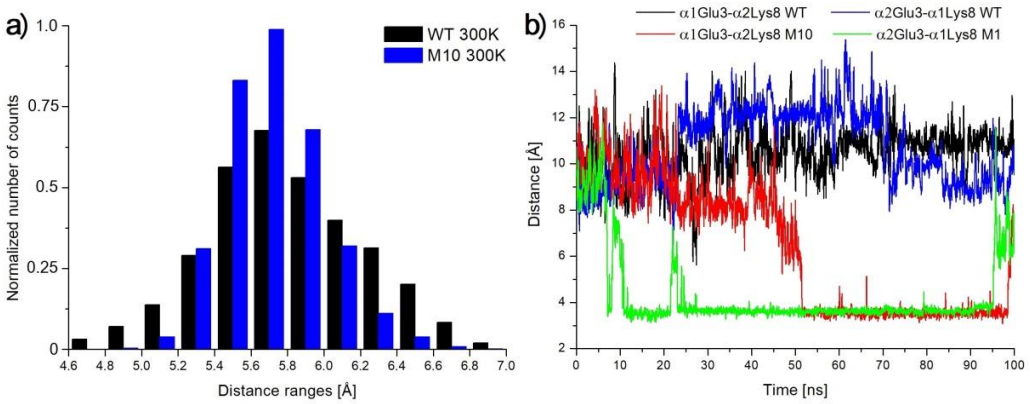

Fig. 9. Influence of αI5P mutation on *Pt*NHase. Histogram of distances between Cα atoms of αAsn4 and αArg7, showing loop rigidity (a), and the effect of loop rigidity on αGlu3-αLys8 salt bridge formation (distances between bridge-forming groups) (b). Source [H4].

Mutations αQ31L, αD92H, βP38L, βF118W, βS130Y and βC218V resulted in the formation of hydrophobic cores (Fig. 10), a close interaction of several hydrophobic amino acids. Previously, it has been shown that removal of water from the protein interior, by the formation of exactly such hydrophobic cores is used in evolution by thermophilic bacteria to increase the thermostability of proteins [64-66].

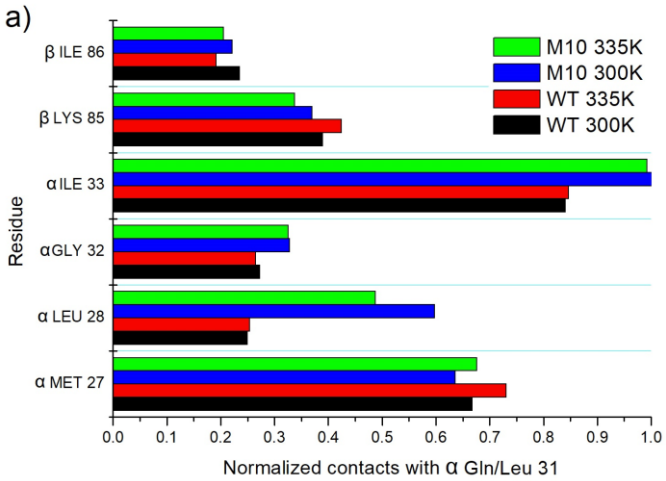

Fig. 10. Plot showing the increased hydrophobic interactions of amino acid 31 of the α-chain. The M10 variant clearly increases hydrophobic interactions with αLeu28 and αIle33. Source [H4].

The αT18Y and βC189N mutations result in the formation of new hydrogen bonds, which has an effect similar to the mutations proposed in the paper [H3].

The introduction of all of the above mutations caused the protein to exhibit much higher thermostability.

In the manuscript, I explained the reasons for the increased catalytic activity of the M10 variant. I docked the substrate i.e. nicotinonitrile to both *Pt*NHase variants. The docking was carried out to an equilibrated structure obtained after 20 ns of simulation at 300K. By using such structures, all amino acids had time to adjust their position in response to the changes introduced by the mutations. It turned out that the increased catalytic activity of the M10 variant of *Pt*NHase, in addition to the improved stability of the enzyme, could be influenced by the better positioning of the substrate (nicotinonitrile) relative to the cobalt ion. The cobalt-nitrile distance (from the catalyzed nitrile) was 5.9 Å for the native protein, and 5.4 Å for the M10 variant (see Fig. 11), resulting in easier activation of the enzymatic reaction. The different orientation of the amino acid, βTrp118 and αGln89, also resulted in a slightly higher substrate-protein interaction energy in the M10 variant than in the native protein (-4.98 kcal/mol vs. -4.18 kcal/mol).

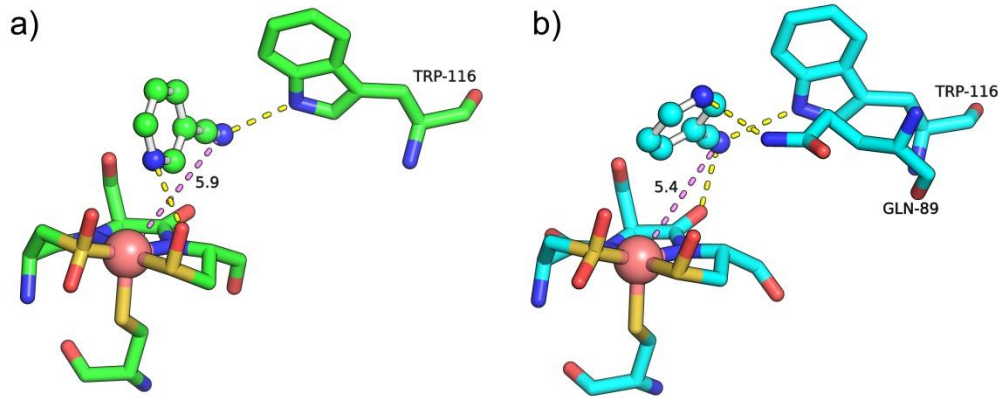

Fig. 11. Result of docking of nicotinonitrile to native PtNHase (a) and M10 variant (b). Source [H4].

Another method of enhancing the thermostability of proteins is the introduction of protein linkers, i.e., additional protein fragments. This is done, for example, to increase the interaction surface area of two subunits, to introduce new hydrogen bonds, salt bridges or hydrophobic cores. This approach was used in the next study, in which α and β chains were merged, as in the publication [59]. In this case, however, several types of long protein linkers characterized by different sizes, flexibility and formation of secondary structures were used. In China, the thermostability and catalytic activity were experimentally tested as to the effect of three types of linkers of different lengths on *Pp*NHase (pdb code 3QXE). 1, 4 or 8 repeats were introduced: the EAAAK sequence (helical linker, enzymes with these linkers were named in the manuscript A1, A4, and A8, respectively), the GGSG sequence (flexible linker, NHases named in the manuscript B1, B4, B8, respectively), the PA sequence (rigid linker, named in the manuscript C1, C4 and C8, respectively), and the results of improving thermostability were published in the manuscript [H5]. It turned out that all protein variants have slightly increased catalytic activity. The activity and thermostability are summarized in Table 1 in [H5]. It was noted that with the number of linker sequence repeats, thermostability increases, regardless of the type of linker. The most stable variant turned out to be *Pp*NHase with eight repeats of the EAAAK sequence (A8). To investigate the reason of increased thermostability of A8, B8 and C8 variants, dimer models were prepared on the base of the crystallographic structure of 3QXE (they correspond to αββα tetramers, since β and α chains (exactly in that order) were joined by linkers to form one long protein chain). This was not a trivial task, since no homology modeling tool could predict the structure of the linkers. Therefore, the sequences of the final and initial fragments of the β and α chains

(from/to the first amino acid of the defined secondary structure) were added to the linker sequences and their structures were predicted using the QUARK (*de novo folding*) tool [67], All generated linker models with NHase fragments were docked to the 1IRE tetramer using the ClusPro 2.0 server (protein-protein docking) [68], next missing protein fragments were added using the PRIME tool from the Schrödinger package. In this way, I obtained models for analyzing static structures. The native *Pp*NHase (3QXE) and the generated models are shown in Figure 12.

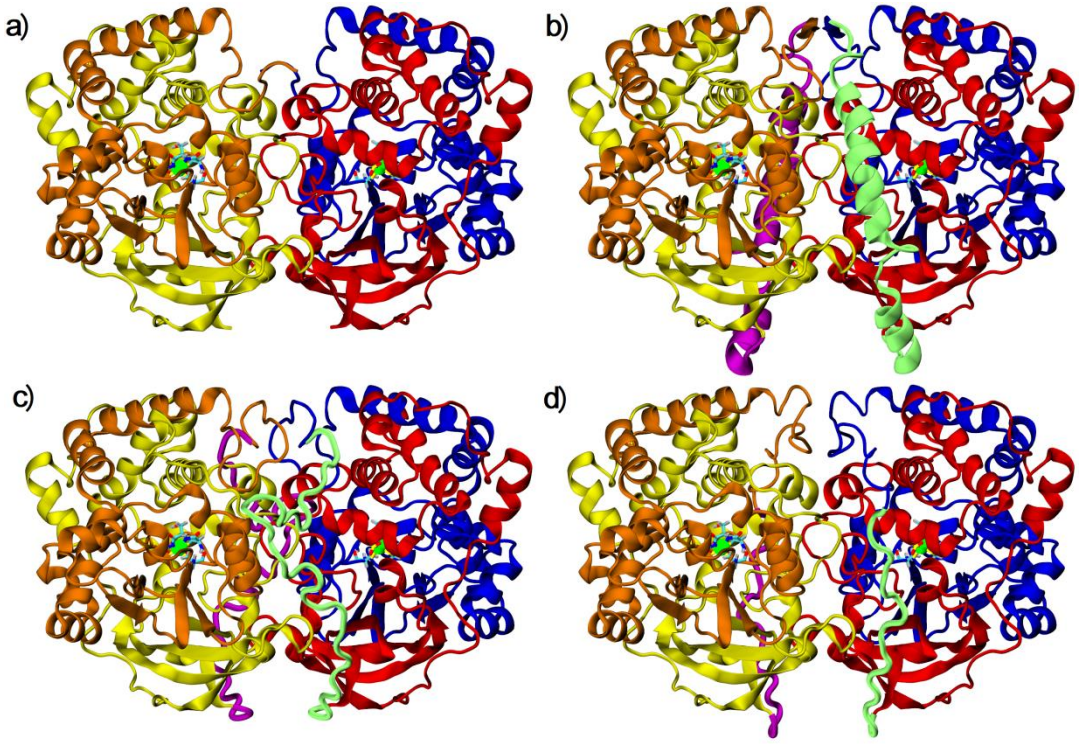

Fig. 12. *Pp*NHase models: native (3QXE) (a), variant A8 (b), variant B8 (c) and C8 (d). Source [H5].

Analysis of the models showed that introducing new hydrogen bonds enhanced the thermostability of the system, an increased interaction area of dimers, and, in the case of variant A8, the formation of 8 new salt bridges.

In order to examine the best A8 variant in detail, I performed molecular dynamics simulations for it and the native protein at two different temperatures of 300K and 335K. Analysis of obtained trajectories showed the reasons for the increased thermostability. First, in the native protein, beginnings a denaturation of the α-chain (Fig. S1 in the supplement [H5]) what could be seen in the simulations with increased temperature. It can be immediately seen in the values of the RMSD plots (Fig. 6 in [H5]). The whole protein was stabilized, as shown by analysis of RMSF plots (Fig. 7 in [H5]). Similar to the studies published in [H4], here I also observed stabilization of

the structure of the active site and the two arginines interacting with it (Fig. 8 in [H5]). In addition, I observed a significant increase in the interaction surface area of the two *Pp*NHase subunits (Fig. S2 in the [H5] supplement), an increase in the number of hydrogen bonds in the protein (Fig. S3 in the [H5] supplement) and between the two subunits (Fig. S4 in the [H5] supplement). Similar to the static structure, the number of salt bridges was increased in the dynamic structure (Table S4 in supplement [H5]). All these factors positively affect the thermostability of the A8 *Pp*NHase variant.

Examples of several different ways to increase the thermostability of NHase are shown above. After that, to demonstrate the generality and universality of my computational approach, I successfully attempted to improve the enzyme sucrose phosphorylase (SPase, EC 2.4.1.7). This enzyme catalyzes sucrose degradation to D-fructose and α-D-glucose 1-phosphate [2]. D-fructose is used in the food industry, while α-D-glucose 1-phosphate is used in the cosmetics industry as an emollient - a substance that acts as a softener, moisturizer, lubricant and regenerative agent.

Again, the industrial application of SPase is limited by its low thermostability. Although the reaction itself is not exoenergetic, bioreactors are quickly polluted with microorganisms due to the presence of sugars in the reaction [27]. A high-efficient SPase derived from the organism *L. mesenteroides* ATCC 12,291 (*Lm*SPase) was used to study thermostability enhancement, and the results were published in the article [H6] [H6].

*Lm*SPase has not been crystallized so far, so there was a need to generate a homology model of this protein. Initially, due to low sequence similarity to crystallized SPases, the I-TASSER tool [69] was used to predict the spatial structure of *Lm*SPase. I used this model as input to the FireProt server [62]. This means that a similar approach was used to predict more thermostable enzymes as in the case of the research published in the paper [H4]. In this case, the FireProt server generated quite a large number of single mutations, and to reduce the experimental workload, I decided to limit the number of changes introduced according to the approach used in the paper [H3]. Based on the results of molecular dynamics simulations, only those sites with the lowest stability were selected. Because the crystallographic structure of a SPase similar to *Lm*SPase was solved in the meantime, I decided to create a more accurate model using the SwissModel server [51]. I confirmed the better quality of the new model using the Verify3D tool [53]. Based on the predictions of the FireProt

server and from the analysis of the calculated RMSF values of the native *Lm*SPase homology model (Fig. 1 in [H6]), 10 potential variants were selected: I31F, T152G, N158C, T219L, A232M, N249A, G252L, T263L, S360A, and Q453G, which were verified experimentally. Four of them, I31F, T219L, T263L, and S360A, were found to have enhanced thermostability and/or activity (but none of them had worse thermostability). A variant having all four mutations was also tested in a Chinese laboratory. It was named Mut4. Unfortunately, it showed a slight worse thermostability than the best T219L variant.

I studied the reasons for the increased stability of the WT, T219L, and Mut4 variants based on MD simulations at 300K and 335K. Trajectory analysis showed that the WT protein at 335K preserved its initial structure much worse (large RMSD values, Fig. 4 in [H6]). Other variants, independently of the temperature set in the simulation, kept their spatial structure better (or similarly) as the native variant (Fig. 4 in [H6]). Regions of the protein in the amino acid ranges 128-133, 307-327, 227-357, and 328-399 became less flexible, as shown by RMSF analysis (Fig. 5 in [H6]).

Contact analysis showed that changing tyrosine to leucine at position 219 creates a hydrophobic core near the catalytic center, namely the amino acids Asp196 and Glu237, and stabilizes this region (see Fig. S3a and S3b in the supplement [H6]). Similar things happen in enzymes of extremophilic organisms [70]. The formation of a hydrophobic core could be observed in both the T219L and Mut4 variants. Analysis of the contacts of the other modified amino acids in the Mut4 variant showed that they do not change their interactions with the surrounding amino acids as significantly (Fig. S3c-e in the supplement [H6]) and thus the introduction of additional three mutations slightly distant from the active site than position 219 does not result in additional improvements in thermostability.

The above four examples of research on increasing enzyme thermostability show that theoretical computational biophysics methods can significantly contribute in designing more thermostable enzymes, which is much needed in modern biotechnology. In my research, I used several different approaches, such as merging two amino acid chains of enzymes and introducing point mutations [H3], using bioinformatics tools in [H4] and [H6], or inserting additional amino acids to increase the interaction surface of enzyme subunits [H5]. In the case of the investigations published in [H4] and [H6], molecular dynamics simulations were used to reduce the

number of mutations that were introduced, so they had a substantial contribution in designing new and improved biotechnological enzyme variants. In all of these works ([H3][H4][H5][H6]), MD simulations helped to elucidate the reasons for the increased thermostability of enzymes. All this leads to a better understanding of the mechanisms of thermostability enhancement, and a similar approach will surely be used in other enzymes.

Designing selectivity of biotechnological enzymes.

The production of specific chemical compounds in chemical technology processes is often not possible, despite tremendous advances in the field, as indicated by the 2022 Nobel Prize in Chemistry given to Carolyn R. Bertozzi, Morten P. Meldal, and K. Barry Sharpless for so-called "click chemistry." Often it is almost impossible to obtain the set compounds, especially the simpler ones. An example is the selective formation of only one optical isomer or performing the reaction of only one functional group in a compound containing two or more identical functional groups. This problem has been overcome through evolution by living organisms, which have "learned" to catalyze selected optical isomers in a racemic solution of a substrate.

In the two studies presented below and the publications [H7] and [H8] presenting the results of these studies, we have shown that it is possible to successfully modify a naturally occurring enzyme to perform reactions for only selected compounds or functional groups.

Having two enantiomers in the racemate, it is difficult to catalyze the reaction of only one of them using chemical technology. The situation is similar with the enantiomers of nitriles. Although NHases catalyzing selected optical isomers of nitriles are known (i.e., they exhibit so-called stereoselectivity) [34, 71], it was not possible to find an NHase catalyzing the hydration of a specific isomer from the racemate of a chemically important compound - mandelonitrile, where an amide with a specific chirality would then become an intermediate in the formation of important drugs [72, 73].

In order to design an NHase having catalytic activity towards one enantiomer of mandelonitrile, an NHase from the organism *Rhodococcus rhodochrous* J1 (according to the publication [H7] L-NHase) was selected. Since the L-NHase was not crystallized to build its spatial structure I used the SwissModel tool [51]. To determine the amino acids to be mutated, I initially docked S-mandelonitrile and

R-mandelonitrile to the L-NHase. Since the starting point was the L-NHase homology model, I docked the ligands to the NHase after 5 ns of MD simulation. Similar to other cases, I performed channel analysis with the CAVER program [46] and, using steered molecular dynamics (SMD), pulled out both enantiomers from the protein interior along the designated channel. Analysis of SMD trajectories identified the largest steric hindrance during ligand pulling and allowed us to determine the amino acids responsible for forming hydrogen bonds with mandelonitrile (Fig. S3 and Tables S2 and S3 in the supplement [H7]). Using this approach, four crucial amino acids affecting the catalysis of mandelonitrile were determined. These were βPhe37, βAla41, βLeu48, and βPhe51. Other amino acids were omitted from the mutation studies to avoid modifying sites essential for catalytic activity. Analysis of the catalytic activity of all possible mutational variants at these four positions (saturated mutagenesis) allowed us to select the variant with the best stereoselectivity, i.e. βF37H (Table 1 in [H7]). It turned out in the experiments that this variant in the formation of S-mandelamide is characterized by 96.8% enantiomeric excess, which means that mainly S-mandelonitrile is catalyzed. In addition, the βF37H variant has a similar efficiency to the native variant. To explain why the βF37H variant shows such significant stereoselectivity towards S-mandelonitrile, I docked both substrates to the βF37H variant as well (again after 5 ns of balancing the system with MD) and performed SMD simulation calculations of pulling out both enantiomers from the βF37H L-NHase.

Analysis of SMD trajectories showed that a higher force is required to “pull out” R-mandelonitrile from βF37H L-NHase than for native protein or S-mandelonitrile from both L-NHase variants. This is mainly caused by the presence of two hydrogen bonds of R-mandelonitrile at the same time: with βHis37 and αGln90 (Fig. 13).

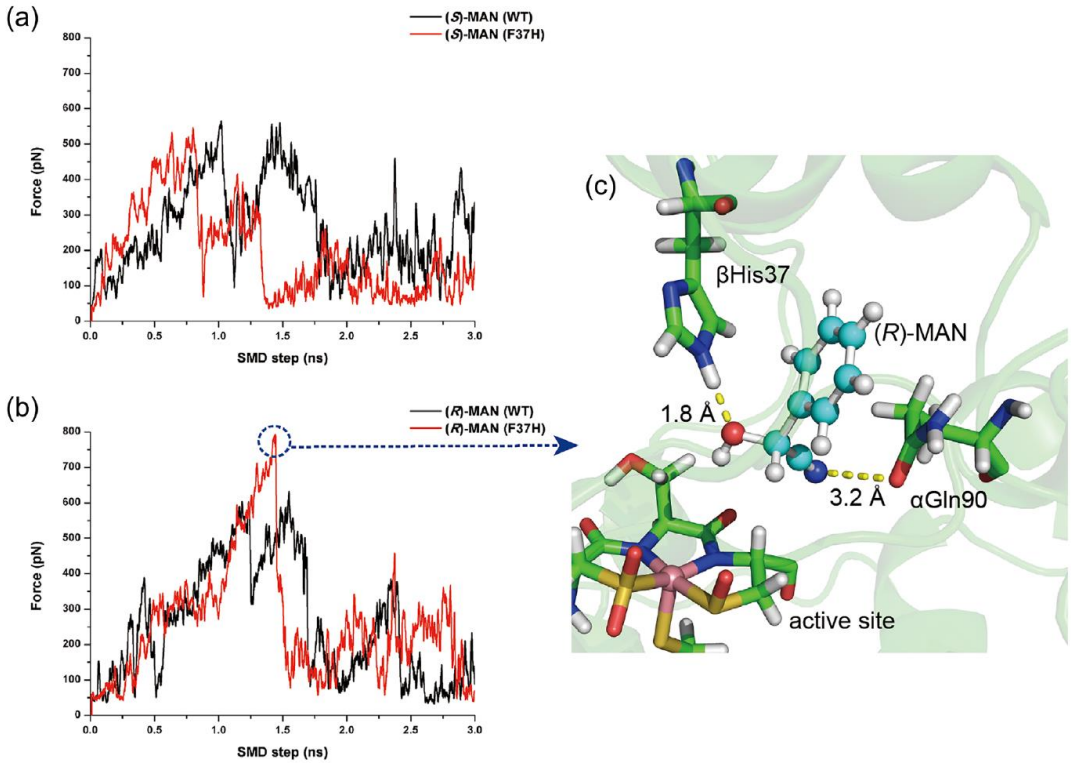

Fig. 13. Plots of the force applied to the ligand during SMD simulations of L-NHase native and βF37H. In panel (a), forces are plotted during the pulling out of S-mandelonitrile, on panel (b) of R-mandelonitrile. Panel (c) shows the main cause of high forces during the pullout of R-mandelonitrile. Source [H7].

With the help of docking, I checked what effect the βF37H mutation has on the orientation of the two enantiomers toward the active site (Figs. 14a, 14b, 14c and 14d). It appeared that the substitution of phenylalanine for histidine caused a slight change in the orientation of the side group of the modified amino acid, and as a result, the rotation of the neighboring phenylalanine 51 from the β-chain. In the native protein, the orientation of both these amino acids causes both S-mandelonitrile and R-mandelonitrile dock in such a way that the nitrile group of the substrate is close to the catalytic center, i.e. at a distance from the cobalt ion of 5.0 Å (Fig. 14a) and 5.4 Å (Fig. 14b), respectively. The change in the position of both amino acids due to the mutation causes, a different orientation of the R-mandelonitrile with respect to the catalytic center shifting the nitrile group to a distance of 7.6 Å (Fig. 14d), preventing the chemical reaction, S-mandelonitrile, on the other hand, can be positioned close enough to the catalytic center to allow the catalytic reaction to be carried out. The nitrogen-cobalt distance, in this case, is 5.3 Å (Fig. 14c). The change in position of the amino acids Phe/His37 and Phe51 and the effect on the orientation of R-mandelonitrile is shown in Figure 14e.

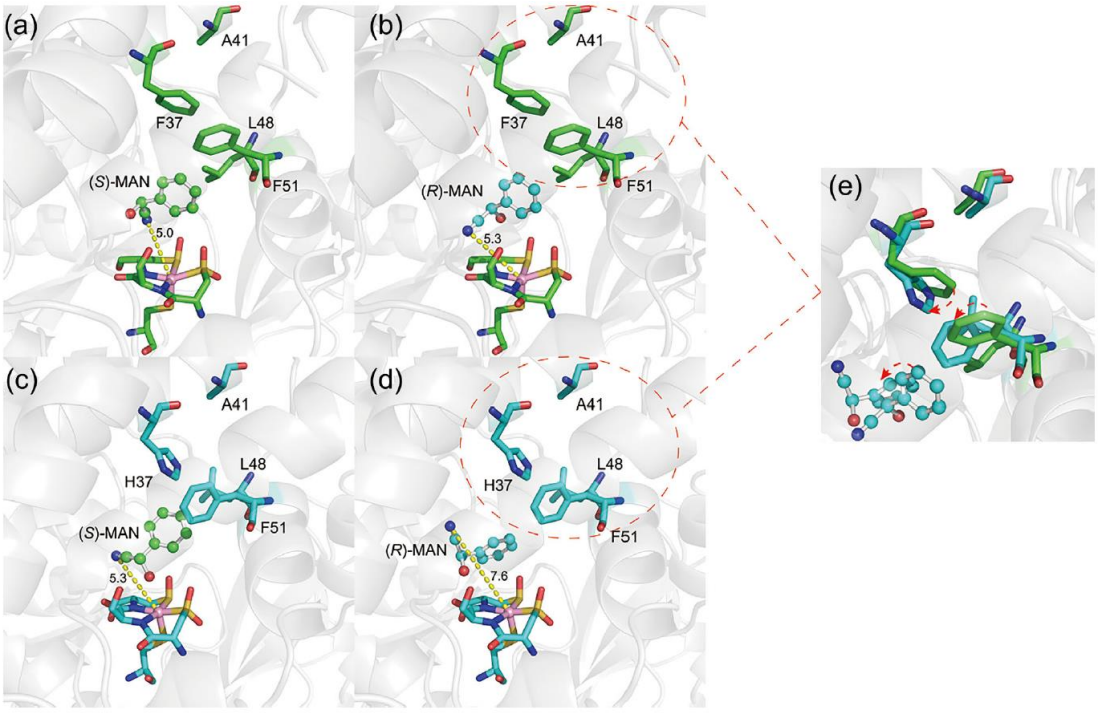

Fig. 14. Results of docking of S-mandelonitrile (a) and R-mandelonitrile (b) to native L-NHase. Docking of S-mandelonitrile and R-mandelonitrile to βF37H L-NHase is shown in panel (c) and (d), respectively. Panel (e) shows a comparison in the orientation of Phe/Leu37 and Phe51 and the effect of this change on the positioning of docked R-mandelonitrile. Source [H7].

Equally difficult as performing the reaction for the chosen enantiomer is to carry out the reaction of only one functional group in compounds containing two identical functional groups. However, it is possible in enzymes to make such a reaction, as we have shown in a study of the stereoselectivity of L-NHase (the same as in the publication [H7]) towards four dinitriles: adiponitrile and malononitrile (aliphatic dinitriles, abbreviations introduced are ADN and MAN, respectively) and terephthalonitrile and phthalonitrile (aromatic dinitriles, abbreviations introduced are TPTN and PTN, respectively) (see Scheme 1 in [H8]). The results of the regioselectivity studies were published in [H8].

When the reaction is carried out using classical chemical technology, only diamides can be obtained from dinitriles. For the chemical and pharmaceutical industries, however, nitrilamides, i.e. derivatives in which only one functional group is hydrated, are very important. A good example of this is 5-cyanovaleramide (5-CVAM), which is formed by hydration of only one nitrile group from ADN. This compound is an important intermediate in the production of herbicides [74] and pharmaceuticals [19].

In the following, I will present the main results of the regioselectivity of L-NHase against ADN only. Identical methodology was used for the other three dinitriles.

The L-NHase model used in this study was the same as in the publication [H7]. I obtained the list of amino acids involved in substrate orientation by docking all ligands to the spatial structure of the protein undergoing 5 ns MD simulation. Based on these results, I proposed the following amino acids for mutations potentially changing regioselectivity: βLeu48, βPhe51, βTyr68, and βTrp72. By saturation mutagenesis, Prof. Zhou's group made all possible NHase variants at the designated positions and tested their catalytic activity against ADN. It was found that two L-NHase variants, βY68T and βW72Y catalyze ADN mainly to 5-CVAM and in a small extent (about 2-3%) to adipamide (diamide, abbreviated as ADAM, Table 2). To check whether better regioselectivity could be obtained, another round of all possible variants was done for the βY68T and βW72Y variants at positions 72 and 68, respectively. It turned out that the βY68T/βW72Y double mutant does not give diamide at all, only 5-CVAM. To explain this regioselectivity, I docked both ADN and 5-CVAM to the L-NHase βY68T/βW72Y. I also docked the same compounds to the native variant of L-NHase (Fig. 15; always docking to the enzyme structure obtained after 5 ns of MD simulation of a particular variant identical to the work in [H7]).

Docking allowed me to elucidate the reasons of increased selectivity of the mutants. ADN docked to the native L-NHase variant has both nitrile groups close to the cobalt ion (5.04 Å and 5.53 Å, respectively, Fig. 15a). Docking ADN into the βY68T/βW72Y variant, shows that one of the nitrile groups can hydrate (4.09 Å, Fig. 15b). In this way, 5-CVAM can be formed. In order for the hydration reaction of 5-CVAM to occur, it must rotate at the catalytic center, or exit the enzyme and again get close to the cobalt ion (which is more likely). In the case of the WT protein, the amide

Table 2. Regioselectivity of L-NHase towards ADN, expressed as % of the product obtained after enzymatic reaction of ADN racemate. Source [H8].

Wariant enzymu	5-CVAM	ADAM
WT	0	100
Y68T	97,6	2,4
W72Y	96,8	3,2
Y68T/W72Y	100	0

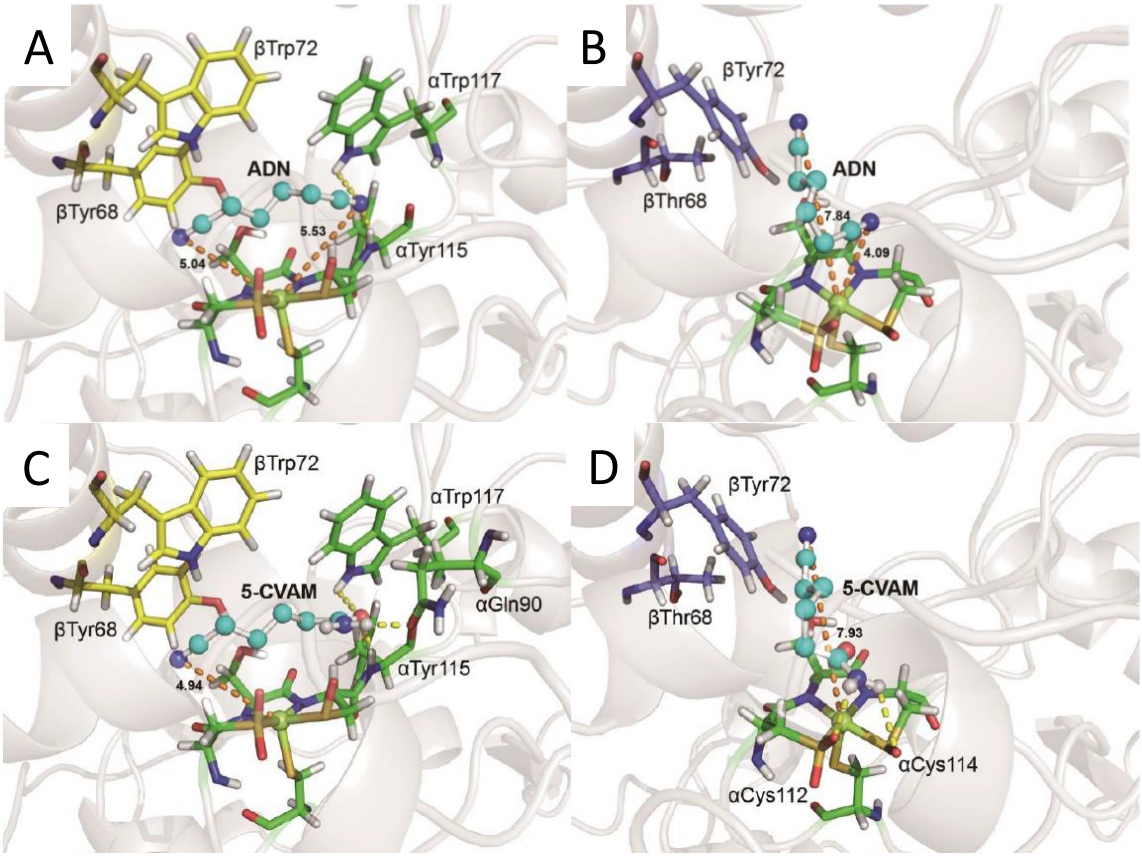

Fig. 15. Results of ADN docking to native L-NHase panel (a) and βY68T/βW72Y L-NHase variant (b). Panels (c) and (d) show the results of docking 5-CVAM to the WT enzyme and the βY68T/βW72Y variant, respectively. Source [H8].

group forms hydrogen bonds with αTrp117 and αGln90, and the nitrile group is oriented towards the cobalt ion (4.94 Å, Fig. 15b).The introducing two mutations βY68T and βW72Y changes the orientation of the amino acids near the active site in such a way that the amide group begins to forms hydrogen bonds with the posttranslationally modified cysteines, resulting that the nitrile group is located always far from the catalytic center. (7.93 Å, Fig. 15d). This causes the hydration reaction of 5-CVAM not to occur, and ADAM (diamide) is not produced.

Making appropriate use of the tools of theoretical molecular biophysics, I designed enzymes that exhibit stereoselectivity or regioselectivity. With these new proteins, previously unknown, it is possible to obtain drug or herbicide intermediates cheaply and in an environmentally friendly way. The analysis schemes I performed using the methods of theoretical computational biophysics proved to be useful not only in elucidating the reasons of increased selectivity, but also in the process of designing improved enzyme variants, making it possible to determine a narrow group of amino

acids for modification, saving the work time and financial resources of the experimental group.

Summary.

In the researches that resulted in the publications included in the habilitation achievement, it was shown that the use of fundamental methods of theoretical computational biophysics, based on well-known principles of Newtonian dynamics, thermodynamics or electrostatics, allows to improve useful enzymes. This approach is, by its nature, highly interdisciplinary, integrating fundamental concepts from disciplines like chemistry, biotechnology, and computer science into biophysical inquiry.

In publications [H1], [H2] as well as in [H4], it was shown that using experimental biotechnology in cooperation with computational biophysics, it is possible to design and isolate a more active enzyme and, importantly, to explain at the molecular level why the improved properties take place. Computational efforts, combined with in-depth structural analysis, have helped to raise the problem of enzyme enhancement to a more rational and physically based level. The articles [H3], [H4], [H5] and [H6] showed that using the tools of theoretical biophysics, it is possible to design enzymes that are more thermostable, which is very important for industry in practical use. The last two papers, [H7] and [H8], showed that the synergy of computational biophysics and experimental biotechnology makes it possible to modify enzymes in such a way that they catalyze only the reactions the researcher wants to indicate. Without computational contribution, this would be very difficult, if not impossible.

The above studies mainly focused on the non-standard enzyme nitrile hydratase. It is worth mentioning that this is an innovative approach, since, generally, molecular modeling tools do not allow to study non-standard proteins (i.e., having posttranslationally modified amino acids or coenzymes). Only expert understanding of the principles of molecular dynamics programs, in-depth knowledge of the laws of physics used in the computational method, its limitations, the ability of programming, as well as a basic knowledge of biology and chemistry, provide a chance to efficiently investigate such proteins and improve them. I am confident that studying the secrets of catalytic activity, thermostability and selectivity of non-standard enzymes requires specialized protocols, continuous expert control of the obtained results, in other words, advanced knowledge is necessary. Scientists with such knowledge in Poland are few,

and from the information I have, no one applies it to biotechnological enzymes. The situation is similar in the world – the well-known docking and molecular dynamics methods are mostly used for standard enzymes. However, due to the very high difficulty of using theoretical computational biophysics methods for non-standard enzymes, it is very rarely used in these cases. My researches carried out in close cooperation with two Chinese scientific teams demonstrate that using theoretical computational biophysics methods for non-standard enzymes is possible. Developed and published analysis methods can be applied to similar problems by all interested scientific teams worldwide. Our articles and experience, plus more and more powerful computers, therefore open up new research possibilities.

4.2.3. Other threads and future research.

I have undertaken other research additional to the methods of improving biotechnological enzymes during my scientific career. These mainly focused to use molecular dynamics methods to explore the properties of biomolecules.

The first example is the study of the nanomechanics of proteins containing cysteine knots. Together with Prof. Marek Cieplak, applying a non-standard method of molecular dynamics, steered molecular dynamics (SMD), we explained why in proteins having such knots one can differentiate between biomolecules that require a very high force to unfold the protein, and those in which this force is much lower. We compared the results to classical proteins that do not contain cysteine knots. The results of the study were published in [P1]. The same SMD method was used in the manuscript [P2], which elucidated the mechanism of the unfolding of modular proteins containing so-called Ig (Immunoglobulin like) domains. The SMD method was used also to model a DNA molecule, explaining the presence of a previously unknown plateau (an area of biopolymer “flow” at a constant force) in some DNA stretching experiments using atomic force microscopy. In our opinion, this work is still underappreciated, rarely cited, and contains the world’s first observation of unusual DNA behavior at very high tension. Simply said, experiments in this force regime are very difficult and only a few groups can obtain such measurement data from AFM.

A further example of the efficient application of theoretical molecular biophysics methods is the study of nitrile hydratase activators. These proteins are necessary for the incorporation of the cobalt ion into the active site of the NHase and probably for making posttranslational modifications. I determined the 3D structure of these

activators and explained the differences in their activity. In addition, we showed that NHase activators have ATPase properties (articles [P5] and [P10]).

I used different theoretical methods in the publications [P3] and [P7]. Quantum calculations of a series of molecules made it possible, in the case of the work [P3], to elaborate a statistical method for analyzing infrared spectra. In the paper [P7], on the other hand, the spectra of a series of dyes containing the azobenzene motif were characterized in detail in collaboration with the experimental groups.

In the future, I intend to further apply theoretical computational biophysics methods not only to biotechnological enzymes, but also to biosensors. Together with Prof. Zhemin Zhou's group, we plan to modify a naturally occurring molecular complex that detects acetamide in such a way that it will be able to detect specific amides, using fluorescent proteins and so-called anti-terminators. So far, it has been possible to develop such a system that detects multiple amides [75], The main goal, however, is to modify this enzyme by introducing several variants of protein detectors to detect different amides. As a result, different amides could be detected even with the naked eye, in effect sensing different NHase products (thanks to fluorescent proteins that illuminate in different colors). Similarly, with Prof. Zhemin Zhou's group, we want to modify the receptor that detects GABA. Recently, with my master's student Magdalena Wlodkowska, we modified the GABA receptor in such a way that it began to detect butyric acid instead of GABA (unpublished data). In the future, we want to design new variants detecting other simple amino acids together with the Chinese group.

Another research stream will be improving enzymes that produce food ingredients. Currently, together with Prof. Xianzhong Chen's group, we have designed a more efficient variant of the HpaB enzyme that produces the natural antioxidant hydroxytyrosol (an ingredient in olive oil). We plan to begin research on the efficient enzymatic formation of polyols, which are natural sweeteners used in the food industry. Polyols are very valuable for those suffering from overweight and diabetes because they are not absorbed from the digestive system and are sweet.

In the near future, I also plan to start a collaboration with Dr. Shangyuan Sang of Ningbo University, Ningbo, China, which will aim to search for detectors of non-fresh seafood (such as fish).

4.2.4. References

H1. Dong Ma†, Zhongyi Cheng†, Łukasz Pepłowski†, Laichuang Han, Yuanyuan Xia, Xiaodong Hou, Junling Guo, Dejing Yin, Yijian Rao*, Zhemin Zhou*; *Insight into the broadened substrate scope of nitrile hydratase by static and dynamic structure analysis*. Chemical Science **13**(28) 8417-8428 (2022);

H2. Junling Guo, Julia Berdychowska, Qianpeng Lai, Yiwei Meng, Zhongyi Cheng*, Łukasz Pepłowski*, Zhemin Zhou*; *"Toolbox" construction of an extremophilic nitrile hydratase from Streptomyces thermoautotrophicus for the promising industrial production of various amides*. International Journal of Biological Macromolecules **221** 1103-1111 (2022);

H3. Yuanyuan Xia, Łukasz Pepłowski, Zhongyi Cheng, Tianyi Wang, Zhongmei Liu, Wenjing Cui, Michihiko Kobayashi*, Zhemin Zhou*; *Improving the thermostability and catalytic efficiency of the subunit-fused nitrile hydratase by semi-rational engineering.* ChemCatChem **10**(6) 1370-1375 (2018);

H4. Zhongyi Cheng, Yao Lan, Junling Guo, Dong Ma, Shijin Jiang, Qianpeng Lai, Zhemin Zhou*, Łukasz Pepłowski*; *Computational design of nitrile hydratase from Pseudonocardia thermophila JCM3095 for improved thermostability*. Molecules **25**(20) 1-18 (2020);

H5. Junling Guo, Zhongyi Cheng, Julia Berdychowska, Xiaonan Zhu, Lingling Wang, Łukasz Pepłowski*, Zhemin Zhou*; *Effect and mechanism analysis of different linkers on efficient catalysis of subunit-fused nitrile hydratase*. International Journal of Biological Macromolecules 181 444-451 (2021);

H6. Yuanyuan Xia, Xiaoyu Li, Linli Yang, Xiaozhou Luo, Wei Shen, Yu Cao, Łukasz Pepłowski*, Xianzhong Chen*; *Development of thermostable sucrose phosphorylase by semi-rational design for efficient biosynthesis of alpha-D-glucosylglycerol*. Applied Microbiology and Biotechnology **105**(19) 7309-7319 (2021);

H7. Zhongyi Cheng, Łukasz Pepłowski, Wenjing Cui, Yuanyuan Xia, Zhongmei Liu, Jialei Zhang, Michihiko Kobayashi, Zhemin Zhou*; *Identification of key residues modulating the stereoselectivity of nitrile hydratase toward rac-mandelonitrile by semi-rational engineering.* Biotechnology and Bioengineering **115**(3) 524-535 (2018);

H8. Zhongyi Cheng, Wenjing Cui, Yuanyuan Xia, Łukasz Pepłowski, Michihiko Kobayashi*, Zhemin Zhou*; *Modulation of nitrile hydratase regioselectivity towards dinitriles by tailoring the substrate binding pocket residues*. ChemCatChem **10**(2) 449-458 (2018);

1. Nagasawa, T., K. Ryuno, and H. Yamada, *Nitrile hydratase of Brevibacterium R312--purification and characterization.* Biochem Biophys Res Commun, 1986. **139**(3): p. 1305-12.
2. Goedl, C., et al., *Sucrose phosphorylase: a powerful transglucosylation catalyst for synthesis of α-D-glucosides as industrial fine chemicals.* Biocatalysis and Biotransformation, 2010. **28**(1): p. 10-21.
3. Sheldon, R.A. and D. Brady, *Green Chemistry, Biocatalysis, and the Chemical Industry of the Future.* ChemSusChem, 2022. **15**(9): p. e202102628.
4. Cabrera-Barjas, G., et al., *Chapter 39 - Food biotechnology: Innovations and challenges*, in *Future Foods*, R. Bhat, Editor. 2022, Academic Press. p. 697-719.
5. Hauer, B., *Embracing Nature's Catalysts: A Viewpoint on the Future of Biocatalysis.* ACS Catalysis, 2020. **10**(15): p. 8418-8427.
6. Steinwand, M.A. and P.C. Ronald, *Crop biotechnology and the future of food.* Nature Food, 2020. **1**(5): p. 273-283.
7. Chen, J., et al., *Microbial transformation of nitriles to high-value acids or amides.* Adv Biochem Eng Biotechnol, 2009. **113**: p. 33-77.

8. Becker, O.M., et al., *Computational Biochemistry and Biophysics*. 2001: Taylor & Francis.
9. MacKerell Jr., A.D., et al., *All-atom empirical potential for molecular modeling and dynamics Studies of proteins.* J Phys Chem B, 1998. **102**: p. 3586-3616.
10. Verlet, L., *Computer "experiments" on clasical fluids. Thermodynamical properties of Lenard-Jones molecules.* Phys. Rev., 1967. **159**: p. 98-104.
11. Swope, W.C., et al., *A computer simulation method for the calculation of equilibrium constants for the formation of physical clusters of molecules: Application to small water clusters.* J. Chem. Phys., 1982. **76**: p. 637-649.
12. Mackerell, A.D., Jr., M. Feig, and C.L. Brooks, 3rd, *Extending the treatment of backbone energetics in protein force fields: limitations of gas-phase quantum mechanics in reproducing protein conformational distributions in molecular dynamics simulations.* J Comput Chem, 2004. **25**(11): p. 1400-15.
13. Peplowski, L., K. Kubiak, and W. Nowak, *Mechanical aspects of nitrile hydratase enzymatic activity. Steered molecular dynamics simulations of Pseudonocardia thermophila JCM 3095.* Chemical Physics Letters, 2008. **467**(1): p. 144-149.
14. Sousa, S.F., P.A. Fernandes, and M.J. Ramos, *Protein-ligand docking: current status and future challenges.* Proteins, 2006. **65**(1): p. 15-26.
15. Khan, F.I., et al., *Current updates on computer aided protein modeling and designing.* Int J Biol Macromol, 2016. **85**: p. 48-62.
16. Morris, G.M., et al., *Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function.* Journal of Computational Chemistry, 1998. **19**(14): p. 1639-1662.
17. Peplowski, L., K. Kubiak, and W. Nowak, *Insights into catalytic activity of industrial enzyme Co-nitrile hydratase. Docking studies of nitriles and amides.* J Mol Model, 2007. **13**(6-7): p. 725-30.
18. Song, L., et al., *High resolution X-ray molecular structure of the nitrile hydratase from Rhodococcus erythropolis AJ270 reveals posttranslational oxidation of two cysteines into sulfinic acids and a novel biocatalytic nitrile hydration mechanism.* Biochem Biophys Res Commun, 2007. **362**(2): p. 319-24.
19. Cheng, Z., Y. Xia, and Z. Zhou, *Recent Advances and Promises in Nitrile Hydratase: From Mechanism to Industrial Applications.* Frontiers in Bioengineering and Biotechnology, 2020. **8**(352).
20. O'Leary, N.A., et al., *Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation.* Nucleic Acids Res, 2016. **44**(D1): p. D733-45.
21. Huang, W., et al., *Crystal structure of nitrile hydratase reveals a novel iron centre in a novel fold.* Structure, 1997. **5**(5): p. 691-9.
22. Kobayashi, M. and S. Shimizu, *Metalloenzyme nitrile hydratase: structure, regulation, and application to biotechnology.* Nat Biotechnol, 1998. **16**(8): p. 733-6.
23. Kobayashi, M., T. Nagasawa, and H. Yamada, *Enzymatic synthesis of acrylamide: a success story not yet over.* Trends Biotechnol, 1992. **10**(11): p. 402-8.
24. Hann, E.C., et al., *5-Cyanovaleramide production using immobilized Pseudomonas chlororaphis B23.* Bioorg Med Chem, 1999. **7**(10): p. 2239-45.
25. Ryuno, K. and T. Nakamura, *Biocatalyst process: Enzymatic transformation of nitrile compounds and the application.* Journal of Synthetic Organic Chemistry Japan, 2003. **61**(5): p. 517-522.
26. Wieser, M., et al., *Low-molecular-mass nitrile hydratase from Rhodococcus rhodochrous J1: purification, substrate specificity and comparison with the analogous high-molecular-mass enzyme.* Fems Microbiol. Lett., 1998. **169**(1): p. 17-22.
27. Haki, G.D. and S.K. Rakshit, *Developments in industrially important thermostable enzymes: a review.* Bioresour Technol, 2003. **89**(1): p. 17-34.
28. Zeymer, C. and D. Hilvert, *Directed Evolution of Protein Catalysts.* Annu Rev Biochem, 2018. **87**: p. 131-157.

29. Qu, G., et al., *The Crucial Role of Methodology Development in Directed Evolution of Selective Enzymes.* Angew Chem Int Ed Engl, 2020. **59**(32): p. 13204-13231.
30. Jiang, W. and B. Fang, *Synthesizing Chiral Drug Intermediates by Biocatalysis.* Appl Biochem Biotechnol, 2020. **192**(1): p. 146-179.
31. Patel, R.N., *Biocatalysis for synthesis of pharmaceuticals.* Bioorg Med Chem, 2018. **26**(7): p. 1252-1274.
32. Snajdrova, R., et al., *Nitrile biotransformation by Aspergillus niger.* Journal of Molecular Catalysis B-Enzymatic, 2004. **29**(1-6): p. 227-232.
33. Martinkova, L. and V. Kren, *Nitrile- and amide-converting microbial enzymes: Stereo-, regio- and chemoselectivity.* Biocatalysis and Biotransformation, 2002. **20**(2): p. 73-93.
34. Prepechalova, I., et al., *Purification and characterization of the enantioselective nitrile hydratase from Rhodococcus equi A4.* Appl Microbiol Biotechnol, 2001. **55**(2): p. 150-6.
35. Robert, K., *Biocatalysis in Organic Synthesis. Science of Synthesis, Vol. 1–3. Edited by Kurt Faber, Wolf-Dieter Fessner and Nicholas J. Turner.* Angewandte Chemie International Edition, 2015. **54**(43): p. 12547-12547.
36. Thomas, S.M., R. DiCosimo, and A. Nagarajan, *Biocatalysis: applications and potentials for the chemical industry.* Trends in Biotechnology, 2002. **20**(6): p. 238-242.
37. Berman, H.M., et al., *The Protein Data Bank.* Nucleic Acids Res, 2000. **28**(1): p. 235-42.
38. Shen, J.D., et al., *Structural insights into the thermostability mechanism of a nitrile hydratase from Caldalkalibacillus thermarum by comparative molecular dynamics simulation.* Proteins, 2021.
39. Liu, J., H. Yu, and Z. Shen, *Insights into thermal stability of thermophilic nitrile hydratases by molecular dynamics simulation.* J Mol Graph Model, 2008. **27**(4): p. 529-35.
40. Chen, J., et al., *Improving stability of nitrile hydratase by bridging the salt-bridges in specific thermal-sensitive regions.* J Biotechnol, 2012. **164**(2): p. 354-62.
41. Pei, X., et al., *Evidence for the participation of an extra alpha-helix at beta-subunit surface in the thermal stability of Co-type nitrile hydratase.* Appl Microbiol Biotechnol, 2018. **102**(18): p. 7891-7900.
42. Zoete, V., et al., *SwissParam: a fast force field generation tool for small organic molecules.* J Comput Chem, 2011. **32**(11): p. 2359-68.
43. Yu, W., et al., *Extension of the CHARMM General Force Field to sulfonyl-containing compounds and its utility in biomolecular simulations.* J Comput Chem, 2012. **33**(31): p. 2451-68.
44. Yamaki, T., et al., *Cloning and sequencing of a nitrile hydratase gene from Pseudonocardia thermophila JCM3095.* Journal of Fermentation and Bioengineering, 1997. **83**(5): p. 474-477.
45. Miyanaga, A., et al., *Crystal structure of cobalt-containing nitrile hydratase.* Biochem Biophys Res Commun, 2001. **288**(5): p. 1169-74.
46. Jurcik, A., et al., *CAVER Analyst 2.0: analysis and visualization of channels and tunnels in protein structures and molecular dynamics trajectories.* Bioinformatics, 2018. **34**(20): p. 3586-3588.
47. Ashkenazy, H., et al., *ConSurf 2016: an improved methodology to estimate and visualize evolutionary conservation in macromolecules.* Nucleic Acids Res, 2016. **44**(W1): p. W344-50.
48. Bennett, R.P., et al., *A Novel Ebola Virus VP40 Matrix Protein-Based Screening for Identification of Novel Candidate Medical Countermeasures.* Viruses, 2020. **13**(1).
49. Bastea, L.I., et al., *Sangivamycin and its derivatives inhibit Haspin-Histone H3-survivin signaling and induce pancreatic cancer cell death.* Sci Rep, 2019. **9**(1): p. 16588.
50. Piersma, S.R., et al., *Arginine 56 mutation in the beta subunit of nitrile hydratase: importance of hydrogen bonding to the non-heme iron center.* J Inorg Biochem, 2000. **80**(3-4): p. 283-8.
51. Waterhouse, A., et al., *SWISS-MODEL: homology modelling of protein structures and complexes.* Nucleic Acids Res, 2018. **46**(W1): p. W296-w303.
52. Colovos, C. and T.O. Yeates, *Verification of protein structures: patterns of nonbonded atomic interactions.* Protein Sci, 1993. **2**(9): p. 1511-9.

53. Eisenberg, D., R. Lüthy, and J.U. Bowie, *VERIFY3D: assessment of protein models with three-dimensional profiles.* Methods Enzymol, 1997. **277**: p. 396-404.
54. Luthy, R., J.U. Bowie, and D. Eisenberg, *Assessment of protein models with three-dimensional profiles.* Nature, 1992. **356**(6364): p. 83-5.
55. Sharma, S., et al., *Chapter 17 - Thermostable Enzymes for Industrial Biotechnology*, in *Advances in Enzyme Technology*, R.S. Singh, et al., Editors. 2019, Elsevier. p. 469-495.
56. Ward, O.P. and M. Moo-Young, *Thermostable enzymes.* Biotechnol Adv, 1988. **6**(1): p. 39-69.
57. Rigoldi, F., et al., *Review: Engineering of thermostable enzymes for industrial applications.* APL Bioeng, 2018. **2**(1): p. 011501.
58. Prasad, S. and T.C. Bhalla, *Nitrile hydratases (NHases): at the interface of academia and industry.* Biotechnol Adv, 2010. **28**(6): p. 725-41.
59. Xia, Y., et al., *Construction of a subunit-fusion nitrile hydratase and discovery of an innovative metal ion transfer pattern.* Sci Rep, 2016. **6**: p. 19183.
60. Leaver-Fay, A., et al., *ROSETTA3: an object-oriented software suite for the simulation and design of macromolecules.* Methods Enzymol, 2011. **487**: p. 545-74.
61. Brodkin, H.R., et al., *Evidence of the participation of remote residues in the catalytic activity of Co-type nitrile hydratase from Pseudomonas putida.* Biochemistry, 2011. **50**(22): p. 4923-35.
62. Musil, M., et al., *FireProt: web server for automated design of thermostable proteins.* Nucleic Acids Res, 2017. **45**(W1): p. W393-w399.
63. Tavernelli, I., S. Cotesta, and E.E. Di Iorio, *Protein Dynamics, Thermal Stability, and Free-Energy Landscapes: A Molecular Dynamics Investigation.* Biophysical Journal, 2003. **85**(4): p. 2641-2649.
64. Panja, A.S., B. Bandopadhyay, and S. Maiti, *Protein Thermostability Is Owing to Their Preferences to Non-Polar Smaller Volume Amino Acids, Variations in Residual Physico-Chemical Properties and More Salt-Bridges.* PLoS One, 2015. **10**(7): p. e0131495.
65. Saelensminde, G., Ø. Halskau, Jr., and I. Jonassen, *Amino acid contacts in proteins adapted to different temperatures: hydrophobic interactions and surface charges play a key role.* Extremophiles, 2009. **13**(1): p. 11-20.
66. Kumar, S., C.J. Tsai, and R. Nussinov, *Factors enhancing protein thermostability.* Protein Eng, 2000. **13**(3): p. 179-91.
67. Xu, D. and Y. Zhang, *Ab initio protein structure assembly using continuous structure fragments and optimized knowledge-based force field.* Proteins, 2012. **80**(7): p. 1715-35.
68. Kozakov, D., et al., *The ClusPro web server for protein-protein docking.* Nat Protoc, 2017. **12**(2): p. 255-278.
69. Yang, J., et al., *The I-TASSER Suite: protein structure and function prediction.* Nat Methods, 2015. **12**(1): p. 7-8.
70. Hait, S., et al., *Finding the generalized molecular principles of protein thermal stability.* Proteins, 2020. **88**(6): p. 788-808.
71. Vejvoda, V., et al., *Biotransformation of heterocyclic dinitriles by Rhodococcus erythropolis and fungal nitrilases.* Biotechnol Lett, 2007. **29**(7): p. 1119-24.
72. Zheng, R.-c., Y.-g. Zheng, and Y.-c. Shen, *Amidase catalyzed kinetic resolution of racemic mandelamide to (R)-mandelate by Delftia tsuruhatensis ZJB-05174.* Journal of Biotechnology, 2008. **136**: p. S368.
73. Lohse, O. and C. Spondlin, *Efficient preparation of (R)- and (S)-2-amino-1-phenylethanol.* ORGANIC PROCESS RESEARCH & DEVELOPMENT, 1997. **1**(3): p. 247-249.
74. Weitz, H.-M., R. Fischer, and F.J. Broecker, *Manufacture of 6-aminocaproamide*. 1978, Google Patents.
75. Yan, Q., et al., *Development of an auto-inducible expression system by nitrogen sources switching based on the nitrogen catabolite repression regulation.* Microb Cell Fact, 2022. **21**(1): p. 73.

5. Presentation of significant scientific or artistic activity carried out at more than one university, scientific or cultural institution, especially at foreign institutions

- In 2011, I completed a 2-month internship at the R&D department of Adamed Sp. z o.o., located in Pienkowo. During the internship, I searched for new small-molecule dual inhibitors of mdm2-p53 and mdm4-p53 interactions, showing anticancer activity in sarcomas, lymphomas and leukemias. Due to the commercial application of these inhibitors, the results obtained are confidential.
- Since 2016, I have been working closely with Prof. Zhemin Zhou's group from the School of Biotechnology, Jiangnan University, Wuxi, China. Since 2018, thanks to my own initiative, this is official cooperation between universities (Resolution No. 36 of the NCU Senate dated 27.02.2018). In cooperation with Prof. Zhou's group, I had 4 short-term visits to Jiangnan University (about 3 months in total). During the visits, I gave 4 invited lectures to the staff and doctoral students of the School of Biotechnology, Jiangnan University, and led 3 workshops on molecular modeling techiques for the doctoral students. In May 2019, three scientists from Jiangnan University visited NCU. The collaboration resulted in 10 joint publications related to the study and modeling of the properties of the biotechnological enzyme nitrile hydratase catalyzing toxic nitriles to useful amides. I have a dual affiliation (NCU and Jiangnan University) in one of these publications. Since 2019, I have also been collaborating with Prof. Xianzhong Chen's group from the School of Biotechnology, Jiangnan University modeling more thermostable versions of the enzyme sucrose phosphorylase, which degrades complex sugars into simpler ones for further use in the food and cosmetic industries. This collaboration has resulted in 2 scientific publications. Recently, I started work with Prof. Chen to enhance the speed of the enzyme (HpaB), which produces valuable ingredient in the food industry hydroxytyrosol.
- List of researchers (or group leaders) outside NCU with whom I collaborate (or have collaborated):
  - Prof. Zhemin Zhou, Jiangnan University, Wuxi, China;
  - Prof. Xianzhong Chen, Jiangnan University, Wuxi, China;
  - Prof. Michihiko Kobayashi, University of Tsukuba, Tsukuba, Japan;
  - Prof. Marek Cieplak, Institute of Physics, PAS, Warsaw, Poland;

6. Presentation of teaching and organizational achievements as well as achievements in popularization of science or art

6.1. Didactic achievements

I teach classes for undergraduate and graduate students, as well as for doctoral students.

Classes after obtaining a doctoral degree, which I coordinate and which I prepared from scratch (the number of hours carried out by me is indicated in parentheses; the number of hours carried out refers to the status as of September 30, 2022. Classes started in the 2022/2023 academic year are not included):

- Computer aided drugs design (laboratory 30h; total 270h completed)
- Biological and medical databases (lecture 15h + laboratory 15h; completed in total: lecture 90h, laboratory 90h)
- Reliability theory (seminar 15h; total 120h completed)
- Bio-nanomaterials (multiple lecturers; lecture 20h; total 20h completed)
- Physics in computer simulation and computer modeling (multiple instructors; lecture 15h, laboratory 15h; completed in total: lecture 45h laboratory 135h)

Classes after obtaining a doctoral degree that I am/was not coordinating (the number of hours taken by me is indicated in parentheses):

- General physics for informatics part 1 (30h exercises; a total of 150h completed)
- General physics for informatics part 2 (30h exercises; 30h total completed)
- Procedural programming (30h laboratory; 330h total completed)
- Programming languages (lab 30h; completed in total 60h)
- Laboratory of numerical problems (multiple instructors; lab 8h; completed a total of 48h)
- Molecular dynamics (lab 30h; completed 30h in total)
- Internet services and cloud computing (exercises 15h; completed 15h in total)
- Elements of physics part 2(exercises 30h; completed in total 60h)
- Elements of physics part 1(exercises 30h; completed in total 120h)
- Introduction to UNIX (lab 30h; completed in total 60h)
- Elements of physics (exercises 30h + laboratory 45h; completed in total exercises 330h, laboratory 90h)
- Elementary physics (exercises 36h; completed in total 180h)
- Programming and numerical methods (laboratory 30h; completed in total 30h)
- Computer laboratory II - programming and numerical methods (laboratory 30h; completed a total of 120h)

- Molecular dynamics methods - Computer modeling of bio-molecules (multiple instructors; lab 10h; completed 10h total)
- Introduction to Bioinformatics (multiple instructors; exercises 10h + lab 15h; completed a total of exercises 10h + lab 15h)
- Physical Laboratory I (laboratory 45h; completed a total of 45h)
- Selected applications of computer science (multiple instructors; lab 15h; completed a total of 15h)
- System of personal assistance (conversion 15h; completed a total of 60h)

I prepared teaching materials for the class:

- Computer aided drugs design. I have divided the materials into two parts. The first part is a presentation about theoretical basics (introduction to biology, introduction to drug chemistry, discussing algorithms) the second part is a detailed instruction on using advanced molecular modeling software.
- Teaching materials for students are available online for Physics in Computer Simulation and Computer Modeling (for the part conducted by me). Materials are prepared for both lecture (presentation) and exercises (modeling projects and how to solve them).
- Biological and medical databases in the form of presentations.
- Reliability theory seminar in the form of presentation.
- Bio-nanomaterials (presentation).
- Internet services and cloud computing (presentation and instruction on how to use virtual machines).
- Materials for the class Numerical Problems Lab (for the part led by me) involving the writing of a molecular dynamics code from scratch, taking into account such physical values as: change of position of atoms (Verlet’s velocity algorithm), determination of velocity as a function of temperature and mass of atoms, determination of acceleration of atoms based on the force acting calculated from van der Waals interactions and electrostatic interactions.

I prepared materials and led classes for foreign doctoral students as part of the summer school “Let's meet at NCU! - International Summer and Winter Intensive Educational Meetings at Nicolaus Copernicus University in Torun" organized by NCU and funded by the SINAKER/NAWA program. For two groups of doctoral students, I conducted a 5-hour class entitled "How to find an inhibitor. Docking of ligands to proteins". Classes in English.

I work with students at all stages of their studies. I supervise or have supervised graduate theses:

- PhD candidates at NCU (associate supervisor)::
  - Dr. Rafal Jakubowski (defense of his doctoral degree in 2016, PhD in physical sciences)
  - Julia Berdychowska, MSc (beginning of PhD studies in 2019, doctoral studies at the Doctoral School of Exact and Natural Sciences, physical sciences)
  - Angelika Klimek, MSc (beginning of PhD studies in 2018, Interdisciplinary Doctoral School Academia Copernicana, biological sciences)
- Master's theses at NCU:
  - Mr. Patryk Muzalewski (Master's thesis started in 2022)
  - Ms. Magdalena Wlodkowska (Achieved the title of "The best master's thesis in the field of Technical Physics in 2020")
  - Ms. Natalia Dymowska
  - Mr. Mariusz Misia
  - Mr. Patryk Kosiorek
  - Mr. Krzysztof Wierzchowski
  - Mr. Rafał Jakubowski
- engineer's theses at NCU:
  - Mr. Rafał Burzyński
  - Mr. Mateusz Wojciechowski
  - Mr. Jakub Bordewicz
  - Mr. Dominik Sieciński
- bachelor's theses at NCU:
  - Mr. Piotr Skrzyniarz
- I assisted with completing Ms. Lan Yao's master's thesis from Jiangnan University, Wuxi, China.
- In addition, I reviewed 1 master's thesis, 12 engineering theses, and 2 bachelor's theses at NCU.
- In 2018, I was a supervisor in the TAPS program (The Torun Astrophysics / Physics Summer program), in which students from outside NCU (including foreign students) completed 4-week scientific internships. Each student carried out a project under the supervision of their mentor. I led the project entitled: "*Investigation of thermal stability of Nitrile Hydratase enzyme using molecular dynamics simulations*".

6.2. Organization achievements

- In 2019, I obtained for NCU participation in the PROM program - International exchange of doctoral students and academic staff (NAWA funding). I am the coordinator of this program at NCU. The program is established to fund academic exchanges mainly for doctoral students. It is planned to finance 100 foreign visits/trips to/from NCU (research internships, conferences and summer schools). The program budget is PLN 1,175,680. Due to the coronavirus pandemic, the program will end in March 2023.
- Since 2006, I have been a member of the organizing committee of the annual international conference BIT BioInformatics in Torun organized by Faculty of Physics Astronomy and Informatics, NCU and the Polish Bioinformatics Society. The conference is held every year in Torun (17 conferences in total). In 2014, the conference was associated with the congress of SocBiN (Society for Bioinformatics in Northern Europe) members. Since 2015, I have been chairman of the organizing committee. In 2022 I was additionally a member of the program committee of this conference. Every year we have between 50 and 120 participants, of which about 30% are foreign participants.
- In 2010/2011, 2011/2012, 2012/2013, 2013/2014, 2014/2015, 2015/2016, I was a member of the University Recruitment Committee at NCU. In 2016/2017 and 2017/2018 I was the secretary of the University Recruitment Committee at NCU.
- Since 2012, I have been the head of the Graphic Station Laboratory as a part of the Computer Modeling Laboratory.
- In 2011, I designed the equipment of computer labs, including the Computer Modeling Laboratory, as part of the creation of the Center for Quantum Optics at Faculty of Physics Astronomy and Informatics, NCU.
- Since 2011, I have been the "Robotic Digital Library" administrator within the National Laboratory for Quantum Technologies. The library is dedicated to archive data on magnetic tapes.
- In 2013, I co-designed the Graphics Terminal Laboratory and the computer cluster (HPC) as part of the establishment of the Interdisciplinary Center for Modern Technologies (ICNT) at NCU.
- Since 2013, I have been the only administrator of the computing cluster and the Graphics Terminals Laboratory at the ICNT, NCU.
- Since 2004, I have been the administrator of servers, computing clusters and computers of the Theoretical Molecular Biophysics Group, Faculty of Physics Astronomy and Informatics, NCU.

6.3. Achievements that popularize science.

- Participation in the 2019 Torun Festival of Science and Arts. I was invited to participate in an plenary panel discussion (main event) entitled. "Man has not one name", during which human beings were presented from the point of view of a sociologist, theologian, physicist, psychologist, artist, philosopher, chemist and mathematician.
- In 2016-2019, I gave a series of talks on theoretical molecular biophysics to small groups of gymnasium and high school students visiting the Faculty of Physics, Astronomy and Informatics under the programs: "Always on the Wave", "Demonstrations and Workshops at Department of Physics, NCU", "Girls for Science", and "Primus of Pomerania and Kuyavia". In total, I conducted classes for at least 477 students.
- Participation in a two-day science picnic (June 1-2, 2016) organized by the The Mill of Knowledge Center for Modernity. I organized a series of demonstrations on the virtual atomic force microscope.
- Participation in the "Region of Exact Sciences" project in 2014 and 2015. The project's main goal was to equalize the opportunities in gymnasium students from countryside areas by enhancing the educational offer with after-school activities in mathematical and natural sciences. I conducted a series of 50 hours (25 x 2h) of lectures and demonstrations on the subject of the role of theoretical molecular biophysics in medicine for the most talented students from countryside areas of the Kuyavian-Pomeranian Voivodeship from 50 gymnasiums.
- In 2013 and 2014, I conducted workshops as part of the "Regional Physics, Mathematics, Computer Science and Astronomy Groups" program. I conducted workshops for groups of students from 12 schools in the Kuyavian-Pomeranian region.
- Leading talks on theoretical molecular biophysics within the framework of the FAST competition ("Physical and Astronomical Scholarships in Toruń") in 2011-2018.

7. Apart from information set out in 1-6 above, the applicant may include other information about his/her professional career, which he/she deems important.

- Since May 2019, I have been a Member of the Board of Disciplines of Physical Sciences at NCU.
- I published an article titled "Polish-Chinese Nature Improvement" in the magazine "Głos Uczelni" 1(383), January 2018, published by NCU, which is intended to serve a culture-opinion mission, presenting the intellectual, scientific, cultural and sports life of Nicolaus Copernicus University in Toruń.

……..……..…….

(Applicant’s signature)