ANNEX 6 TO THE APPLICATION FOR THE INITIATION OF THE HABILITATION PROCEDURE

The importance of oligomerization for the enzymatic activity of trimeric purine nucleoside phosphorylase - biophysical studies using a class of multisubstrate inhibitors

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1 Name and surname

Beata Maria Wielgus – Kutrowska

2 Scientific degrees

• Master's degree in Physics, specialization Biophysics - Faculty of Physics, University of Warsaw, October 13, 1993, the title of Master's thesis *„Investigation of the interaction of purine nucleoside phosphorylase with nicotinamide riboside by spectroscopic methods"*, supervised by dr hab. Ewa Kulikowska.
• PhD degree in Physics - Faculty of Physics, University of Warsaw, December 6, 1999, the title of the PhD dissertation *"Purine nucleoside phosphorylases - physico-chemical properties and the mechanism of interaction with ligands"*, supervised by prof. David Shugar.

3 Employment and trainings in scientific research laboratories

• From 1993- Department of Biophysics, Institute of Experimental Physics, Faculty of Physics, University of Warsaw:

- 1993 - 1998 - PhD studies,
- 1999 – Technical position,
- 2000 - 2004 - Adjunct, fixed term contract,
- 2004 - Adjunct, permanent contract.

4 Scientific publications in the database Web of Science

• Index # H 9
• Number of publications 33 (+ 3, published in 1996, 2014, 2014, not mentioned in the WoS)
• Number of citations: 292
• The number of citations excluding self-citations: 224

List of publications (36) divided into publications presented as the scientific achievement, the other, found in the Journal Citation Reports, and the other, are placed in the Annex 4

5 Indication of the achievement according to Art. 16 § 2 of the Act on Academic Degrees and Title, and Degrees and Title in Art, 14 March 2003 (Journal of Laws No. 65, item 595 with amendments)

5.1 Title of a scientific achievement

"The importance of oligomerization for the enzymatic activity of trimeric purine nucleoside phosphorylase - biophysical studies using a class of multisubstrate inhibitors”

5.2 List of publications representing the scientific achievement

1. A. Bzowska, G. Koellner, B. Wielgus-Kutrowska, A. Stroh, G. Raszewski, A. Holý, T. Steiner, J. Frank (**2004**) *„Crystal structure of calf spleen purine nucleoside phosphorylase with two full trimers*

in the asymmetric unit: important implications for the mechanism of catalysis" J. Mol. Biol. 342, 1015-1032.

My contribution to this publication was:

- planning and performing of spectroscopic measurements (absorption and fluorescence, shown in Figures 7 and 8A and Table 2B),
- a statistical analysis of the results of spectroscopic measurements,
- preparation of spectroscopic data in the form of figures and table,
- participation in discussions on all of the results presented in the publication.

I estimate my participation for the 20%.

2. B. Wielgus-Kutrowska, A. Bzowska (**2006**) *„Probing the mechanism of purine nucleoside phosphorylase by steady-state kinetic studies and ligand binding characterization determined by fluorimetric titrations"* Biochim Biophys Acta. 1764, 887-902.

My contribution to this publication was:

- performing of all experimental measurements,
- statistical analysis and interpretation of the obtained results,
- preparation of the figures and tables (except Scheme 1),
- participation in the writing and preparation of the final version of the manuscript after reviews.

I estimate my participation for the 80%.

3. B. Wielgus-Kutrowska, J.M. Antosiewicz, M. Dlugosz, A. Holý, A. Bzowska (**2007**) *„Towards the mechanism of trimeric purine nucleoside phosphorylases: stopped-flow studies of binding of multisubstrate analogue inhibitor - 2-amino-9-[2-(phosphonomethoxy)ethyl]-6-sulfanylpurine"* Biophys. Chem. 125, 260-268.

My contribution to this publication was:

- performing of all experimental measurements,
- analysis and interpretation of the results obtained, together with coauthors,
- preparing all figures and tables,
- participation in the writing and final preparation of the manuscript after reviews.

I estimate my participation for the 70 %.

4. K. Breer, A. Girstun, B. Wielgus-Kutrowska, K. Staroń, A. Bzowska (**2008**) *„Overexpression, purification and characterization of functional calf purine nucleoside phosphorylase (PNP)"* Protein Expr. Purif. 61, 122-130.

My contribution to this publication was:

- arranging the purchase of a cDNA library and invitation to the cooperation of the Faculty of Biology, University of Warsaw,
- participation in the design of vectors and the method of the expression of the calf purine nucleoside phosphorylase in *E. coli*,

- supervision on the methodology of purification and on spectroscopic studies of the recombinant PNP, the results of which are presented in Tables 1, 2, 3, 4, 5, and Figures 1, 2, 4, 5, 6.
- participation in the writing and final preparation of the manuscript after reviews.

I estimate my participation for the 30%.

5. K. Breer, L. Glavas-Obrovac, M. Suver, S. Hikishima, M. Hashimoto, T. Yokomatsu, B. Wielgus-Kutrowska, L. Magnowska, A. Bzowska (**2010**) *„9-Deazaguanine derivatives connected by a linker to difluoromethylene phosphonic acid are slow-binding picomolar inhibitors of trimeric purine nucleoside phosphorylase"* FEBS J. 277, 1747-1760.

My contribution to this publication was:

- performing (individually or with the help of technician) the experiments whose results are presented in Figures 2, 3, 4, 7 and , in part, in Table 1,
- data analysis or assistance in the data analysis performed by the PhD student,
- discussion of results of measurements,
- participation in the writing and final preparation of the manuscript after reviews.

I estimate my participation for the 30%.

6. G. Chojnowski, K. Breer, M. Narczyk, B. Wielgus-Kutrowska, H. Czapinska, M. Hashimoto, S. Hikishima, T. Yokomatsu, M. Bochtler, A. Girstun, K. Staron, A. Bzowska (2010) *„1.45 . resolution crystal structure of recombinant PNP in complex with a pM multisubstrate analogue inhibitor bearing one feature of the postulated transition state"* Biochem. Biophys. Res. Commun. 391, 703-708.

My contribution to this publication was:

- designing and assistance in analysis of experiment, whose results are shown in Figure 1,
- discussion of results of experiments,
- participation in the writing and final preparation of the manuscript after reviews.

I estimate my participation for the 15%.

7. K. Breer, B. Wielgus-Kutrowska, A. Girstun, K. Staroń, M. Hashimoto, S. Hikishima, T. Yokomatsu, A. Bzowska (**2010**) *„Overexpressed proteins may act as mops removing their ligands from the host cells: a case study of calf PNP"* Biochem. Biophys. Res. Commun. 391, 1203-1209.

My contribution to this publication was:

- designing a part of the experiments, including those, which confirm the hypothesis explaining some surprising results,
- performing experiments and analysis of data, or assistance in performing and analysis of experiments by PhD student in the case of all results of calorimetric and spectroscopic studies and simulations (Figures 1 - 4, Table 1),
- interpretation of research results, together with collaborators,
- participation in the writing and final preparation of the manuscript after reviews.

I estimate my participation for the 40 %.

8. B. Wielgus-Kutrowska, K. Breer, M. Hashimoto, S. Hikishima, T. Yokomatsu, M. Narczyk, A. Dyzma, A. Girstun, K. Staroń, A. Bzowska, (**2012**) *„Trimeric purine nucleoside phosphorylase: exploring postulated one-third-of-the-sites binding in the transition state”* Bioorg. Med. Chem. 20, 6758-6769.

My contribution to this publication was:

- performing of the experimental investigation, which results are shown in Figures 4, 5, 6,
- planning and assistance in and experimental work of PhD students and data analysis in the case of some presented in the publication research results (Figures 1, 2, 3, Table 2 and 3)
- presentation of research results in the tables and figures,
- interpretation of research results,
- participation in the writing and final preparation of the manuscript after reviews.

I estimate my participation for the 50%.

9. B. Wielgus-Kutrowska, A. Modrak-Wójcik, A. Dyzma, K. Breer, M. Żółkiewski, A. Bzowska (**2014**) *„Purine nucleoside phosphorylase activity decline is related to the decay of the enzyme in the trimeric form”* Arch. Biochem. Biophys. 549, 40–48 (DOI 10.1016/j.abb.2014.03.009).

My contribution to this publication was:

- determination of the idea of the research investigations,
- planning and performing of experimental investigations (including all preliminary tests), the results of which are shown in Table 1 and in Figures 2B, 3, 5, 6 (I have performed preliminary studies for Figures 2 and 4),
- • preparation of research results in the form of Tables and Figures (except Figure 2),
- interpretation of the research results,
- writing and final preparation of the manuscript after reviews.

I estimate my participation for the 70%.

5.3 Overview of the research aim of the papers mentioned and the results achieved

Introduction

As monothematic scientific achievement, a set of publications on the biophysical basis of the function of the broad class of enzymes - trimeric purine nucleoside phosphorylases (PNP) is presented. The studies described in the papers have led to:

- Final conclusion on the phenomenon of communication between the subunits of the PNP from calf spleen, which leads, according to the opinion of some scientists, to a very strong negative cooperativity,
- Characterization of the affinity to PNP of the multisubstrate inhibitors - potential drugs, which can be used to block the activity of purine nucleoside phosphorylase, for example in a prevention of transplant rejection,
- Explanation why the PNP is biologically active only as trimeric molecule, consists of three monomers with the same amino acid sequence.

I performed my research investigations in the group of Prof. Agnieszka Bzowska. Her help in many aspects of the studies can not to be underestimated. Thanks to her earlier investigations with collaborators from the Institute of Crystallography, Freie Universität in Berlin, in particular Getraud Koellner, PhD, became possible to initialize research studies described below. They could be carried out thanks to chemists - initially Prof. Antonin Holý, next the group of Prof. Tsutomu Yokomatsu, and biologists - Prof. Krzysztof Staroń and Agnieszka Girstun, PhD. Valuable contribution to the realization of the studies had graduate students, especially Katarzyna Breer, PhD. Without their participation the time needed to collection of the scientific research results would extend significantly, and some of them might not be obtain at all.

Enzymes oligomerization

A significant part of about 60 000 structures of enzymes deposited in Protein Data Bank is composed of several subunits with identical amino acid sequence. They are called sequential homooligomers. The oligomerization allows proteins to form large structures without increasing of genome size, to build binding sites at interfaces of the subunits, allows the allosteric regulation of enzymes and contributes to greater stability of the oligomer compared with the monomer [Hashimoto K. et al. 2011].

There is known a phenomenon of a different affinity of subunits to ligands for many homooligomeric proteins. In a particular, it can be only one active subunit in the enzyme. Other subunits may exist in a conformation that impedes or prevents the binding of ligands. This may be a result of an arrangement of the protein quaternary structure which takes the form of an asymmetric heterooligomer in the apo conformation, i.e. without bound ligands. In such a structure subunits are never identical. This phenomenon is called „preexistent asymmetry" or „site heterogeneity" [Malhotra O.P. et al. 1987].

According to the second hypothesis, the oligomeric form of apo enzyme is symmetrical. The asymmetry is induced by interaction with ligands and communication between the subunits. This can lead to facilitate (in the case of a positive cooperation) or hinder (in the case of a negative cooperation) binding of the subsequent ligand molecule upon binding the first one.
The negative cooperation can be sometimes so strong that the binding of one molecule inhibitor leads to a complete blockage of the oligomer. This phenomenon is described, for dimers , as a „half-of-the-sites binding" or „half-of-the-sites reactivity" (when a protein has an enzymatic activity), or, for the trimers, as the „third-of-the-sites binding/reactivity".

This fact has been proven for several homooligomers from different sources, for example, thymidylate synthase [Saxl R.L. et al. 2001, Pozzi C. et al. 2012], alkaline phosphatase [Papaleo E. et al. 2013, Cathala G., Brunel C. 1975], glyceraldehyde-3-phosphate [Nagradova N.K. 2002, Song S.Y. et al. 1999]. For others appeared a few reports on this problem. In some cases blocking of the enzyme was found to be an artifact, for example, for the enzyme from a microorganism *Rhodospirillum rubrum* with the dual activity of carboxylase and ribulosobiphosphate oxygenase [Herndon C.S. et al. 1982], for transaldolase from *Escherichia coli* [Schörken U. et al. 1998], and transketolase from *Saccharomyces cerevisiae* [Fiedler E. et al. 2002].

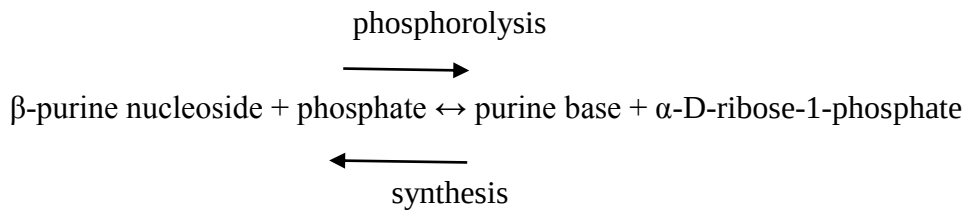
In the vast majority of cases, reports refer to proteins composed of an even number of monomers, for which half of the subunits is switched off after the occupation by ligands of the second half of them. In this case, the interesting seemed to be the research results of Prof. Vern Schramm group, who

suggest the possibility of blockage in certain conditions the entire PNP molecule with an odd number of identical subunits (three) through one molecule of the hypoxanthine (Hx) - the substrate of the nucleoside synthesis reaction, as well as potent inhibitors - Immucillin H and G (ImmH, ImmG). The results are presented in the publications [Kline P.C., Schramm V.L., 1992] and [Miles R.W. et al. 1998]. In another paper they showed the existence of a strong negative cooperativity in the case of Immucillin H with dissociation constants 23 pM for the first molecules bound by trimer , < 5 μM, for the second and ~ 100 mM for the third [Wang F. et al. 2000].

The phenomenon of very strong negative cooperativity in the case of PNP poses many questions. The binding of one molecule blocks practically all oligomer - what is the aim of the phenomenon in which, when the ligand in bound in the one active site, the others become useless? Is this phenomenon observed for other ligands than Hx and ImmH? If yes, how the protein subunits communicate and what are the characteristics feature of the ligands responsible for this effect?

Trimeric purine nucleoside phosphorylases

Purine nucleoside phosphorylases which belong to the class of "low molecular mass", approximately 90 kDa, in an active form are composed of three subunits with identical aminoacids sequence. To this group belong phosphorylases from mammals (calf spleen, human erythrocytes) and from some microorganisms (*Cellulomonas sp.*).
The trimeric purine nucleoside phosphorylases catalyze the reversible phosphorolysis of the glycosidic bond of ribo- and deoxyribonucleosides in the presence of inorganic phosphate according to the scheme:



They are specific for 6-oxonucleosides.

In 1975 Eloize Giblett with collaborators have found that the inactivation of phosphorylase leads to impaired cell-mediated immunity - decline of the number of T cells [Giblett E.R. et al. 1975]. In the case of immune system diseases, such as leukemia, associated with high levels of T-type antibodies, or in situations where the lack of resistance is desired, e.g. organ transplantation, and in many autoimmune diseases, efficient inhibitors of human PNP could be desirable immunosuppresive agents. Studies on the mechanism of the reaction catalyzed by PNP are conducted for many years because of the medical meaning of their results for the design of potent inhibitors of the enzyme.
Three models of enzyme catalysis by trimeric PNP are proposed. All are consistent on two issues concerning 1) the conversion in transition state of the pentose fragment in a positively charged oxocarbenium ion and 2) the crucial role of amino acids Asn243 and Glu201. They differ in the distribution of electron density and type of tautomeric form of the purine ring occurs in a transition state. The oldest hypothesis, which assumes the binding of neutral forms of base and protonation of purine N^7 position during catalysis became the basis for the design of inhibitors that mimic the transition state ligands. The second model assumes the appearance of a negative charge distributed

between N^7 and N^9 purine positions and binding of a negatively charged base at the initial stage of reaction. The third proposes binding of the neutral form of base and the appearance of a rare enol form of purine with negative charge localized around the oxygen O^6.

On the basis of current knowledge could be hypothesized that the potent inhibitors, and immunosuppressive drugs may be the following compounds:

a) the transition state inhibitors – which mimics the ligands in transition state, characterized by the positive charge localized at ribose, and the protonated nitrogen N^7 position of the purine. They imitate of the purine nucleoside-phosphate configuration, which is formed during breaking of the glycosidic bond (C-N). This bound in the transition state inhibitors should be much stronger to be not broken after ligand binding (C-C),
b) multisubstrate inhibitors - compounds which are consisted of a purine derivative, an acyclic chain, and a group containing phosphate (phosphonate or phosphomethylate) which mimic simultaneously two substrates of the reaction catalyzed by PNP: purine base and ribose-1-phosphate (synthesis direction) or purine nucleoside and phosphate (phosphorolysis direction).

A very potent inhibitor of phosphorylases from mammalian sources, the transition state inhibitor, Immucillin H was synthesized recently and its properties were described. It has been registered as a drug (Forodesine, [Al-Kali A., et al. 2010]),

and it is currently in clinical trials. In addition to inhibition constant (23 pM) [Wang F. et al. 2000], according to the authors, ImmH is characterized by a very strong negative cooperativity leading to blocking trimeric PNP molecules after binding of one molecule of inhibitor.

Presentation of the scientific achievement

In the investigations described as a monothematic scientific achievement I have studied the properties of multisubstrate inhibitors, which can became potential drugs. Using these ligands, I have also verified the hypothesis of very strong negative cooperativity („third-of-the-sites binding") for trimeric phosphorylases. The study was based on investigation with application of biophysical methods in relation to complex biological objects - enzymes.
The first publication, which is the starting point for further research is:

A. Bzowska, G. Koellner, B. Wielgus-Kutrowska, A. Stroh, G. Raszewski, A. Holý, T. Steiner, J. Frank (2004) *„Crystal structure of calf spleen purine nucleoside phosphorylase with two full trimers in the asymmetric unit: important implications for the mechanism of catalysis"* J. Mol. Biol. 342, 1015-1032.

We have presented the results of investigation of the communication between the subunits of purine nucleoside phosphorylase from calf spleen, which could accompany the ground state analogs (ligands without the features of the transition state) binding. We have performed the diffraction studies on protein crystals and spectroscopic and calorimetric measurements in solution.

We have determined the three-dimensional structure of the binary complex of PNP with multisubstrate inhibitor - 2,6-diamino-(S)-9-[2-(phosphonomethoxy)propyl]purine ((S)-PMPDAP) at 2.3 Å resolution, and ternary complex of PNP with the inhibitor (S)-PMPDAP and phosphate at 2.05 Å

resolution. It was a first time where two trimers of calf spleen PNP were obtained in asymmetry subunit. Until now, obtained crystals had the monomer in the asymmetric unit and based on the symmetry operations perfectly symmetrical oligomer was built (for human protein trimer has been observed in the asymmetric unit, but with low resolution). The possibility of describing of the oligomeric structure of the enzyme molecule allows to detect possible differences in the three-dimensional structure of the subunits. Such differences would be expected due to „third-of-the-sites binding" events documented for transition-state inhibitors. However, no differences are noted, and binding stoichiometry of three inhibitor molecules per enzyme trimer was observed in the crystal structure. No qualitative differences were also detected between complexes obtained with or without the phosphate, except for the hydrogen bond contact of Arg84 and phosphonate group, which is observed only in the in three of six independent monomers in PNP - inhibitor complex obtained in the presence of inorganic phosphate. Such differences could be the cause of a different stoichiometry of hypoxanthine binding [Kline P.C., Schramm V.L. 1992]. Parallel solution studies using isothermal titration calorimetry (ITC) and spectrofluorimetric titration were consistent with crystallographic data. We determined the stoichiometry of binding of the inhibitor (S)-PMPDAP by PNP. We performed measurements of the activity of different samples of protein (fully activated and partly or totally inactive). Applying a correction connected to the inactivation of PNP we shown that the true stoichiometry in each case is three molecules of the inhibitor per protein trimer. Binding of a ligand is a well-described by model of one type of binding site.

We conclude that:
• multisubstrate inhibitor, an analogue of the ground state ligand, (S)-PMPDAP bounds to the all active sites in the trimer,
• there is no evidence that, in the case of the binding of (S)-PMPDAP by calf PNP, the communication between the subunits of the enzyme leading to negative cooperativity, is observed. Binding of one molecule of the inhibitor does not change the conformation of the protein which may impede the binding of the next inhibitor molecule.

Next publication :

B. Wielgus-Kutrowska, A. Bzowska (2006) *„Probing the mechanism of purine nucleoside phosphorylase by steady-state kinetic studies and ligand binding characterization determined by fluorimetric titrations"* Biochim Biophys Acta., 1764, 887-902,

contains the results of spectroscopic (absorption and fluorescent) studies that have been carried out for the trimeric enzyme from a microorganism *Cellulomonas*.

During the phosphorolysis the glycosidic bond was breaking. The nascent purine base had a different UV absorption spectrum than the parent nucleoside. By monitoring the changes of absorption in chosen wavelength, the progress of the glycosidic bond breaking in the presence of the enzyme, as a function of the ligands concentration (substrates and inhibitors) was observed, in order to determine the value of parameters describing the reaction (Michaelis-Menten constant, inhibition constant , the maximum velocity of the reaction).

In the fluorescent titration method the change in the emission of PNP, due to protein-ligand interaction were monitored, and the values of the association or dissociation constants, and the stoichiometry of the protein-ligand binding were determined.

Using the methods described above I have performed spectroscopic characteristics of the reversible reaction of phosphorolysis for typical and atypical substrates. I discussed the products inhibition, the reaction mechanism (including importance for catalysis of hydrogen bonding N^1 -H ... Glu204) and the deviation from the simple Michaelis- Menten model.

From the point of view of the investigation of communication between the subunits which leads to a strong negative cooperativity, the important fact was that fluorescence titrations data for hypoxanthine (like for guanine) are sufficiently well described by a single binding site model. This means that the trimer has one binding site, or three identical binding sites described by one dissociation constant. Ligand binding stoichiometry indicates the presence of three identical binding sites. Trimer binds three molecules of guanine with and without the presence of phosphate. Value obtained for hypoxanthine in the absence of phosphate was 2.2 ± 0.6 , less than three, but significantly higher than one molecule of ligand per the trimer. (We tried to explain the lower stoichiometry for Hx as a result of certain characteristic of the transition state in the absence of phosphate. Similarly claimed [Kline P.C., Schramm V.L., 1992]).
The publication includes also the description of phosphorolysis inhibition by multisubstrate inhibitors: 2-amino-9-[3-hydroxy-2-(phosphonomethoxy)propyl]-purine ((S)-HPMP-6-Gua), 2-amino-9-[2-(phosphonomethoxy)ethyl]-6-sulfanyl-purine (PME-6-thioGua) and 2,6-diamino-9-[2-(phosphonomethoxy)ethyl]-8-azapurine (PME-8-aza-DAP). Obtained for PME-6-thioGua and PME-8-aza-DAP inhibition constants are 0.16 μM and 0.28 μM, respectively. For PME-6-thioGua a fluorescent titration was done, which proved that the PNP trimer bind 3 inhibitor molecule. We have not observed in this case communication between the subunits of the PNP and negative cooperativity associated with the binding of bisubstrate inhibitors.

Conclusions:
• For ground state inhibitors the binding stoichiometry is three molecules per protein trimer and subunit interaction can be neglected.
• Hx probably can have a transition state features in the absence of phosphate, which cause lower stoichiometry of this ligand binding.

Next, in cooperation with the group of Prof. Antosiewicz we have used the stopped-flow technique to study the communication between the subunits of the PNP. The initialization of the inhibitor binding reaction was made by rapid mixing of solutions from two syringes, one of which contained an enzyme, and the second one – a ligand. The changes in fluorescence above 320 nm (with cut-off filter) excited at 290 nm were monitored. The kinetic transients registered after mixing a protein solution with ligand solutions of different concentrations were simultaneously fitted by several association reaction models using procedure based on numerical integration of the chemical kinetic equations appropriate for given model (developed by Maciej Dlugosz). In these models PNP molecules were treated as objects containing three identical active sites. I have considered models in which, after ligand binding, the protein conformational isomerization took place (the two-step model), or such isomerization did not occur (one-step model). In addition, for each model I assumed the existence or lack of communication

between active sites. I have analyzed data on the assumption that at any time the observed fluorescence is the sum of contributions from all individuals present in the solution: free protein, free ligand and different protein-ligand complexes.

The results of the investigation were published in:

B. Wielgus-Kutrowska, J.M. Antosiewicz, M. Dlugosz, A. Holý, A. Bzowska (2007) *„Towards the mechanism of trimeric purine nucleoside phosphorylases: stopped-flow studies of binding of multisubstrate analogue inhibitor - 2-amino-9-[2-(phosphonomethoxy)ethyl]-6-sulfanylpurine”* Biophys Chem. 125, 260-268,

which contained the results of binding studies of multisubstrate inhibitor - 2-amino-9-[2-(phosphonomethoxy)ethyl]-6-sulfanylpurine (PME-6-thio-Gua) to purine nucleoside phosphorylase from *Cellulomonas sp.* I have concluded, that in contrast to Immucillins, described by Prof. Schramm group, multisubstrate inhibitor without transition state feature do not lead to changes in protein conformation.

- The binding of the inhibitor is well described by a one-step process.
- The negative cooperativity in binding inhibitor was not observed (even the existence of a positive cooperativity was suspected, but the final conclusion requires additional tests.
- The interaction of N^1 -H ... Glu204 is essential for catalysis.

Trimeric purine nucleoside phosphorylase from calf spleen was the most appropriate object for our investigation. It shows a high homology to the PNP from human erythrocytes (sequence identical in 88%, consistent tertiary and quaternary structures, totally conserved the active site and the same reaction mechanism). Calf phosphorylase may be a substitute for non-stable and non-homogeneous human enzyme, and for the exotic PNP from *Cellulomonas sp.* Unfortunately, during our work, commercially available PNP has been withdrawn from sale.

The biophysical techniques require large amounts of material and performing experiments with protein mutants. During the postdoctoral training in the lab of Prof. Patricia Clark I have learned to receive the chosen proteins and their mutants. After return to Poland I have decided to obtain a recombinant calf spleen PNP in Warsaw. Therefore we have bought a cDNA library, initialized the cooperation with the Department of Biology, University of Warsaw and engaged PhD student in the project. In cooperation with the laboratory of Prof. Staroń we have cloned the enzyme, we have developed a two-step method of overexpression and purification with high yield (35 mg protein from 1 liter of bacterial culture, purified to a 92 % of purity) and we have measured the basic properties of the resulting PNP.

All results are presented in the publication:

K. Breer, A. Girstun, B. Wielgus-Kutrowska, K. Staroń, A. Bzowska (2008) *„Overexpression, purification and characterization of functional calf purine nucleoside phosphorylase (PNP)”* Protein Expr. Purif. 61, 122-130.

Recombinant PNP has the same characteristics as the phosphorylase isolated directly from calf spleen: identical sequence and kinetic properties versus substrates (Ino, Guo, m^7Guo). It enhances the fluorescence of guanine, whose binding is well described by the non-interacting site model. In this

situation recombinant protein, overexpressed in *Escherichia coli*, due to the high efficiency of expression and purification, is a good object to verification of the subunits interaction mechanism in the case of the transition state and basic state inhibitors binding. This allowed to use of techniques that require large amounts of material, such as calorimetry. Therefore, the enzyme expressed in bacteria became the basis for further investigation of the „third-of-the-sites binding/reactivity" phenomenon and for searching for potent multisubstrate inhibitors.

The recombinant PNP crystallized under the same conditions as the protein isolated from calf spleen. We have got the crystals which were used to solve the three-dimensional structure of the PNP by X-ray diffraction method. The results were published in the next paper:

G. Chojnowski, K. Breer, M. Narczyk, B. Wielgus-Kutrowska, H. Czapinska, M. Hashimoto, S. Hikishima, T. Yokomatsu, M. Bochtler, A. Girstun, K. Staroń, A. Bzowska (2010) *„1.45 A resolution crystal structure of recombinant PNP in complex with a pM multisubstrate analogue inhibitor bearing one feature of the postulated transition state”* Biochem. Biophys. Res. Commun. 391, 703-708.

We report a crystal structure of recombinant PNP in complex with multisubstrate inhibitor: 9-(5',5'-difluoro-5'-phosphonopentyl)-9-deazaguanine (DFPP-DG) at 1.45 Å resolution. This is the highest resolution published for PNPs so far. The crystals contain the full PNP homotrimer in the asymmetric unit, as in the paper published in 2004. Determination of the structure was the subject of the doctoral thesis of one of the co-authors. I have participated in the spectroscopic studies of inhibitor DFPP-DG, which, like immucillins, has protonated the N^7 purine position, but it has not the positive charge on the ribose ring. This strong inhibitor can be bound with the same K_d (approximately 190 pM) to all three substrate binding sites of the trimeric PNP. We have observed in the crystal structure three molecules of the DFPP-DG bond by trimeric molecule of enzyme. Fluorimetric titrations were consistent with the crystallographic data and showed that the phenomenon of „third-of-the-sites binding" does not occur for this ligand. Because of these and previous results, for which we can not observe the binding of one inhibitor molecule by protein trimer, we propose that the postulated phenomena „third-of-the-sites binding” of immucillins should be rather attribute to the second feature of the transition state, ribooxocarbenium ion character of the ligand or to the coexistence of both features characteristic for the transition state.

Because DFPP-DG was found to be a good inhibitor of mammalian PNP, in the paper :

K. Breer, L. Glavas-Obrovac, M. Suver, S. Hikishima, M. Hashimoto, T. Yokomatsu, B. Wielgus-Kutrowska, L. Magnowska, A. Bzowska (2010) *„9-Deazaguanine derivatives connected by a linker to difluoromethylene phosphonic acid are slow-binding picomolar inhibitors of trimeric purine nucleoside phosphorylase”* FEBS J. 277, 1747-1760,

we described the studies of the mechanism of its binding by calf spleen PNP. The inhibition constant at equilibrium (1 mM phosphate concentration) with calf spleen PNP was shown to be = 85±13 pM (pH 7.0, 25^{o}C) and was in the same range as for the Immucillin H (23 pM). Stopped-flow experiments are most consistent with a two-step binding mechanism:

E + I <=> (EI) <=> (EI)*.

According to this model the first step is the creation of enzyme-inhibitor complex (EI), which, due to conformational changes leading to the formation of strong interaction of the enzyme-inhibitor complex (EI)*. We checked how the presence DFPP-DG in the medium affect the inhibition of the growth of cell lines sensible to inhibition of PNP activity, such as human adult T-cell leukaemia and lymphoma (Jurkat, HuT78 and CCRF-CEM). The inhibitor exhibits weak, but statistically significant, inhibition of the growth of these cell lines. The observed weak cytotoxicity may be a result of poor membrane permeability.

Next, we have measured the interaction of DFPP-DG with the recombinant PNP by izotermic titration calorimetry. The method involves the gradual injection of small amounts of ligand solution into the calorimetric cell with the enzyme solution, at equal time intervals until all binding sites will be filled by the inhibitor. The change of enthalpy caused by ligand binding was the measured.
Titration calorimetry gave initially endothermic signal which changed next into exothermic. Such a phenomenon was not observed in the case of commercially available PNP isolated from calf spleen. In that case only endothermic signal was present. In order to explain the unusual phenomenon the additional studies were performed, and the results, their analysis and interpretation were published in the paper:

K. Breer, B. Wielgus-Kutrowska, A. Girstun, K. Staroń, M. Hashimoto, S. Hikishima, T. Yokomatsu, A. Bzowska (2010) *„Overexpressed proteins may act as mops removing their ligands from the host cells: a case study of calf PNP”* Biochem. Biophys. Res. Commun. 391, 1203-1209.

Analysis of calorimetric titrations of recombinant PNP with selected inhibitors, described in the publication point to the two types of binding sites. Such results could be caused by a negative cooperation, or by different forms of the enzyme. Assuming the existence of two forms of protein (weakly and strongly binding) we made a successful separation of them by affinity chromatography, and, also, by dialysis. For describing of calorimetric measurements performed for the strongly binding form of PNP a model with one binding site was sufficient.

The measurements of absorption spectra confirmed the hypothesis that the part of recombinant enzyme formed a complex with the product of phosphorolysis - hypoxanthine. Free enzyme was the strong binding form of the protein, and the complex enzyme-hypoxanthine was the weak binding form. Strong inhibitor bound with free enzyme with one binding constant. It bound with the complex enzyme-hypoxanthine with another apparent binding constant. Since binding of hypoxanthine is accompanied with a large negative change of the enthalpy, the replacement of this compound by DFPP-DG, characterized by lower binding enthalpy, yields positive heat signal. Hypoxanthine, however, was not present at any purification stage. It naturally occurred only in cells of *E. coli* in which PNP was expressed. It means that the phosphorylase bound the hypoxanthine present in bacterial cells. This ligand remained bound to the protein at all stages of the enzyme purification, affecting the results of the ligands binding measurement.

We showed that the compounds present in the host cells can bind to the enzyme. Similar processes - moping of ligands from the host cells – may take place in the case of other proteins with high overexpression yield.

This finding, in view of our previous studies, suggested that the phenomenon „third-of-the-sites binding" described for the transition state inhibitors does not occur. Therefore, additional studies have been performed for binding by PNP of the Immucillin H, and of the inhibitors characterized by: (1) lack of, (2) one or (3) two features of transition state.

The results have been presented in the publication:

B. Wielgus-Kutrowska, K. Breer, M. Hashimoto, S. Hikishima, T. Yokomatsu, M. Narczyk, A. Dyzma, A. Girstun, K. Staroń, A. Bzowska (2012) ***"Trimeric purine nucleoside phosphorylase: exploring postulated one-third-of-the-sites binding in the transition state"*** **Bioorg. Med. Chem. 20, 6758-6769.**

The studies were conducted using isothermal titration calorimetry, fluorescence titration and genetic engineering. Since fluorescent response for Immucillin H binding by the PNP is insignificant new mutants were constructed with kinetic properties similar to those of wild-type PNP, but with more intense fluorescence response to ligand binding (Phe159Trp-PNP and Phe200Trp-PNP). We have analyzed the kinetic properties of the inhibitors that do not have (DFPP-G, DFTHPEP-H, (S)-PMP-DAP), have one (Hx, Gua, 9-deaza-Gua) or two (DFPP-aza-DG) transition state features. Measurements were done under the same conditions in which the existence of the phenomenon of „third-of-the-sites binding" were reported. We have put a great attention to proper dialysis of hypoxanthine.

The calorimetric measurements have not shown the negative cooperativity - the binding of Immucillin H is consistent with the model of the three ligand molecules binding by the enzyme trimer. Similar results were obtained for fluorimetric titration of PNP by Immucillin H. The same situation occurred in the case of titration of PNP by other inhibitors with transition state features. Simultaneously, we observed an apparent cooperativity between the subunits of the PNP and lower stoichiometry in the case of recombinant enzyme, not fully purified from hypoxanthine.
These results refute the hypothesis of the total trimer PNP inhibition by one molecule of potent inhibitor. I proposed the probable explanation of the reasons for which previous experiments have been misinterpreted.
Binding of Immucillin H generates more heat than binding of Hx, so replacing Hx by Immucillin H gives exothermic contribution into signal. In the experiments we see the change of shape of the titration curve, which leads to incorrect conclusions about two binding sites model describing the results of measurements.
In the case of weak inhibitor binding by the PNP with part of the binding sites occupied by Hx, a model of one binding site appears to be sufficient to describe the experimental data. However, the stoichiometry is lower than one per binding site, because the weak inhibitor does not push out the bound hypoxanthine. Hence, probably, observed in previous experiments (see: B. Wielgus-Kutrowska, A. Bzowska (2006) Biochim Biophys Acta, 1764:887-902), the lower stoichiometry of binding Hx by the enzyme with part binding sites occupied by this ligand.
We checked that in some samples from Sigma absorption at 250 nm was slightly increased. It may indicate that some commercially available samples also contained hypoxanthine.
The ITC results were confirmed for the various ligands (DFPP-G DFPP-DG DFPP, DFPP-aza-DG, Immucillin H, Gua, 9-deazaGua, Hx) using fluorescence titration. In cases where the binding of ligand

results in a little change in fluorescence of wild-type PNP, the tryptophan mutants: Phe200Trp-PNP and Phe159Trp-PNP were used. Fluorescence data analysis confirmed in all cases that the model with one binding site and the binding stoichiometry of one ligand per enzyme subunit is sufficient to describe the titration results.
Since, according to the literature, the binding of one molecule of Immucillin H practically blocked the binding of ligands to other binding sites in the trimeric PNP we decided to check it out. We have prepared a complex of one molecule Immucillin H with PNP trimer. Next we have titrated such a complex by guanine. It was found that the binding of one molecule of Immucillin H does not block binding of other ligands for the remaining binding sites of the trimeric PNP.

- We do not noticed the phenomenon of „third-of-the-sites binding" for a variety of ligands, including these which have two feature of the transition state. Incorrect reports on the existence of this phenomenon may be due to the presence of hypoxanthine (derived from the organism in which the protein was expressed, or due to the employed method of purification) blocking of the part of binding sites.
- We can conclude that in the case of trimeric PNP (from *Cellulomonas sp.*, calf spleen, and, probably, from human erythrocytes) monomers conducting independently the enzymatic reactions.

In this case the question arises: why the PNP is a biologically active as a trimer, since there is no communication between the subunits and each monomer works independently? To answer this question, I decided to do the investigations, the results of which are presented in the following publication:

B. Wielgus-Kutrowska, A. Modrak-Wójcik, A. Dyzma, K. Breer, M. Zolkiewski, A. Bzowska (2014) *„Purine nucleoside phosphorylase activity decline is related to the decay of the enzyme in the trimeric form"* Arch. Biochem. Biophys. 549, 40–48 (DOI 10.1016/j.abb.2014.03.009),

Experiments were conducted by various biophysical techniques including analytical ultracentrifugation, circular dichroism spectroscopy and differential scanning calorimetry.

Analytical ultracentrifugation method consists in the monitoring of biomolecules motion in the centrifugal force field with centrifugal acceleration exceeding the acceleration due to gravity to 290000 times. The sedimentation coefficient describes the move of biomolecules in the liquid, in the centrifugal force field and it is equal to the velocity of the molecule per unit of centrifugal acceleration. This factor is closely connected to the mass, shape and volume of the particles, the temperature, the diffusion coefficient and liquid properties such as viscosity and density. It allows specifying in what form biomolecules (monomer, trimer, and larger complexes) are present in a solution.
Circular dichroism spectroscopy, due to the chirality of biological molecules associated with the content of α-helical and β-sheets structures allows to specify the percentage of the characteristic elements of protein secondary structure.
Differential scanning calorimetry describes the phase changes in the biopolymers initiated by heat delivery and it is useful in studies of protein stability.

We have shown that, that the activity decline (due to protein aging after isolation from cells) of wild type PNP and its two mutants with point mutations in the region of monomer-monomer interface, is

accompanied by a decrease of the population of the trimeric enzyme and an increase of the population of its aggregated form. The data do not indicate a significant population of either folded or unfolded PNP monomers. The enzyme with specific activity lower than the maximal shows a decrease of the helical structure, which can make it prone to aggregation. The presence of phosphate stabilizes the enzyme but leads to a more pronounced aggregation above the melting temperature (T_m).

These results confirmed the importance of oligomerization of trimeric purine nucleoside phosphorylase for the stabilization of the protein. It making possible to play the biological function, since the monomers are not stable in aqueous solutions.

Summary

Performed research investigations led to the revision of the negative cooperativity phenomenon for trimeric PNP using multisubstrate inhibitors. These inhibitors can be helpful for drug design. Together with collaborators:

- I have developed a method of obtaining of recombinant calf spleen PNP and its mutants by overexpression in *E. coli*. Nowadays, we can obtain the protein in sufficient quantities to carry out the biophysical experiments of various types, what is particularly important after withdrawal from the sale of the protein extracted from calf spleen.
- I have describe the properties of new PNP mutants: Phe159Trp-PNP and Phe200Trp-PNP with enhanced fluorescent response to ligand binding.
- I have found a very potent inhibitor of trimeric PNP with inhibition constant comparable with that for Immucillin H - the transition state inhibitor registered as a drug. DFPP-DG, unfortunately, does not penetrate cell membranes, therefore, further studies are needed.
- I have revised the present in the literature hypothesis of very strong negative cooperativity between the subunits of PNP. This phenomenon does not occur. Like other ligands, the hypoxanthine and transition state inhibitors, for which this phenomenon has been suggested, do not block binding of the molecules to the unoccupied binding sites of enzyme.
- I have explained the probable causes of incorrect postulate of the „third-of-the-sites binding" phenomenon. A commonly used method for overexpression of proteins in *Escherichia coli* cells can result in a material which contains trapped ligands of host cells. In our case it was hypoxanthine. Ligand bound to the enzyme also appears during the purification by affinity chromatography. If the investigator is unaware of the presence of protein-bound ligands he may obtain incorrect conclusions of the study. It is important to draw attention to such cause of potential errors in the case of overexpression and purification of other proteins, not only PNP.
- I have used a new biophysical method - analytical ultracentrifugation . Thanks to its use, I found that the PNP is a biologically active protein only as trimer. The role of oligomerization is the stabilization of the native form of this protein.

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6 Other Research Activities

6.1. Introduction

In 1987, after graduating from high school (XIV LO, Warsaw) I began studies at the Faculty of Physics, University of Warsaw. I finished it after 3 years of physics studies and 2.5 years of specialization in the field of Biophysics. Master's thesis *„Investigation of the interaction of purine nucleoside phosphorylase with nicotinamide riboside by spectroscopic methods"* I have written under the supervision of dr hab. Ewa Kulikowska. The Master thesis contained the results of investigation of phosphorolysis of unusual substrate - nicotinamide riboside by purine nucleoside phosphorylase (PNP) from calf spleen and bacteria *Escherichia coli*, using UV-absorption spectroscopy methods.

In 1993, I began PhD studies at the Faculty of Physics, University of Warsaw under the supervision of prof. David Shugar. I have investigated the mechanism of interaction of PNP from calf spleen, *Escherichia coli* and *Cellulomonas sp*. with ligands. In addition to absorption spectroscopy I have begun to use the stationary fluorescence spectroscopy techniques and I have learned how to obtain crystals of biomolecules, proper for diffraction measurements. I was performing the study of physicochemical properties of phosphorylases and interactions with ligands in the Department of Biophysics. In cooperation with the group of prof. Saenger (in particular with Dr. Getraud Koellner and Jan Tebbe), at the Institute of Crystallography, Freie Universität in Berlin, I participated in the project, which aim was to obtain diffraction structure of PNP. The result of several years of work was obtaining the three dimensional structure of the new phosphorylase, proposition of a mechanism for the reaction catalyzed by PNP from *Cellulomonas sp*. (this mechanism also explains the behavior of phosphorylases from mammalian sources) and the characterization of the interaction of phosphorylases from different sources with derivatives of 8-azapurine. The results of my research investigations have been described in the PhD dissertation *„Purine nucleoside phosphorylase - physicochemical properties and the mechanism of the interaction with ligands”.*

After receiving PhD degree in 1999, I have been employed in 2000, as an adjunct for four years. In 2003, I have participated in 11th-month postdoctoral training in the laboratory of Prof. Patricia Clark, at the Department of Biochemistry, University of Notre Dame, U.S.A. In 2004 I was employed as an adjunct (permanent contract) in the Department of Biophysics. Now I work in a group of Prof. Agnieszka Bzowska. In the years 2007 - 2012 I have participated in short-term trainings in group of Prof. Andrzej Sienkiewicz at the Department of Physics, Ecole Polytechnique Federale de Lausanne, Switzerland (2007 and 2010) and in the group of Prof. Michal Zolkiewski at the Department of Biochemistry and Biophysics at Kansas State University in the U.S. A. (2012).

The subject of my research interests include the study of protein-ligand interactions mechanisms, mechanisms of adoption of the correct, biologically active protein structure and description of this three dimensional structure, and the causes of protein aggregation.

This is an interdisciplinary scientific research, where I use modern methods of molecular biophysics, molecular biology and biochemistry, with techniques such as spectroscopic measurements by stopped-flow method, which allow to observe the phenomena occurring in biological systems at the milliseconds time scale, two types of calorimetry: isothermal titration calorimetry and differential scanning calorimetry, circular dichroism spectroscopy, electron paramagnetic resonance and analytical

ultracentrifugation. I learned how to clone recombinant proteins, overexpressed them in bacteria, purify, and construct mutants for the research purpose.

Until 2006 our research group had only one laboratory. We did not have the possibility of proteins purification to a satisfactory degree for spectroscopic studies (such purification we have to perform first, in the laboratory of Prof. Saenger in Berlin, and, next, in the laboratory of Prof. Darżynkiewicz in the Department of Biophysics, University of Warsaw). I have realize that for further research we need develop the research laboratories. After postdoctoral training in the laboratory of Prof. Patricia Clark, together with Prof. Agnieszka Bzowska we began to build new laboratories: spectroscopic laboratory (2006), two biotechnological laboratories (2008), calorimetric laboratory and analytical ultracentrifugation laboratory (2010). I took care of laboratory equipment in an appropriate, professional research instruments (ITC microcalorimeter, a centrifuge, the FPLC station, laminar, autoclave and small scientific appliances). Particularly important was a purchase of the analytical ultracentrifuge within NanoFun consortium, headed by Anna Niedźwiecka, PhD, and Jerzy Trzciński, PhD. When we have applied for funds, in Poland was not any of this type of centrifuge. Now they are three, but only we have an ultracentrifuge equipped with fluorescence detection system. I have participated in trainings on the handling of purchased equipment, organized in the Department of Biophysics, or abroad (e.g. workshop on analytical ultracentrifugation and data analysis at the National Institute of Health, Bethesda, USA). At the same time I cared about the development of our research group. I was a supervisor of master's (4) and bachelor's (4) theses. Three of my four students continue their work in our research group. I coordinated partially the research work of one of doctoral students, for the second I manage the project, in which she performs her doctoral thesis.

As an academic teacher I join academic research with the teaching at the Faculty of Physics, Warsaw University. The giving of lecture „Methods of Molecular Biophysics " keeps me in contact with the newest discovery in the field, in which I want to remain professional. I participate in the popularization of science, by giving the lecture in schools, or in the framework of the Summer School of Physics, writing articles for popular-science magazine „Wiedza i Życie", participating or coordinating the activities of the Festival of Science at the Department of Physics. I was a member of the Scientific Council of the Physics Faculty. I initialized many cooperation with other scientific departments and institutions in Poland and abroad. I was the manager of one and the main contractor for many research projects. I am a co-author of 36 papers published in international journals. My total citation index is 292, and my Hirsch index is 9. The results of our work have been presented by me or PhD students at many international conferences.

6.2. Scientific research after obtaining a PhD degree

Other publications that are not classified as a scientific achievement, published after obtaining a doctoral degree, can be divided into the following sections:

I. Folding and aggregation of Green Fluorescent Protein (GFP) mutants

After postdoctoral training, in 2003, I began investigation on protein folding and competitive process - aggregation. The objects of these studies are mutants of green fluorescent protein (GFP). The unique feature of this biomolecule is the fluorescence in the visible range caused by presence of an unique chromophore, which is built spontaneously by three following amino acids in the protein chain: serine,

tyrosine and glycin, after creating the proper structure of the native protein. GFP is used as a biological marker in biology, medicine and biotechnology. Its discovery, investigations of the properties, and the idea of applications have been appreciated by the Nobel committee - in 2008 three scientists received the Nobel Prize in chemistry. I have described the history of the discovery and unique features of GFP in popular-science article published twice in „Wiedza i Życie" - in May 2009 and in the special issue „Light" in 2011. GFP can be easily attached to any protein and can be expressed with it in the cells. Its fluorescence is a marker describing the distribution, transport, and aggregation of the protein in the cell. However, GFP also tends to aggregate during folding to the native structure and the chromophore formation depends on the presence of reducing agents in the solution.

The preliminary results of our investigations of the folding and aggregation of GFP were described in communication:

B. Wielgus-Kutrowska, P.L. Clark (2004) *„Folding and aggregation of GFP, an anti-parallel beta-barrel protein with a complex strand topology”* Biophys. J., 86 Suppl. 266A-267A.

After formation, the chromophore is very stable and remains intact during protein unfolding, meaning that the GFP unfolding process is not the reverse of the original folding reaction; i.e., the principles of microscopic reversibility do not apply. We have generated the mutant S65T/G67A-GFP, which is unable to form the functional chromophore, with the goal of investigating the folding, unfolding and competing aggregation of GFP under fully reversible conditions. Our studies have been performed in the presence of GdnHCl. The GFP conformation was monitored using intrinsic tryptophan fluorescence, and fluorescence of bisANS (4,4'-Dianilino-1,1'-Binaphthyl-5,5'-Disulfonic Acid). Light scattering was used to follow GFP aggregation. We conclude from these fluorescence measurements, that S65T/G67A-GFP folding is largely reversible. During equilibrium folding, the first step is formation of „molten globule”, prone to aggregation.

The results are described in the paper :

B Wielgus-Kutrowska, M Narczyk, A Buszko, A. Bzowska, P.L. Clark (2007) *„Folding and unfolding of a non-fluorescent mutant of green fluorescent protein”* J. Phys. Condens. Matt. 19, 285223.

GFP is prone to aggregation and formation of its chromophore critically depends on the presence of reducing agents. Therefore we have undertaken spectroscopic kinetic studies of EGFP folding and aggregation as a function of pH, and in the presence of various reducing agents, to study the competition between these two processes. The best conditions for folding of EGFP provides BME (β-mercaptoethanol) as a reducing agent. Aggregation of EGFP depends strongly on pH, and on the concentration of the protein. The careful control experiments must therefore be performed during investigations of proteins fused with EGFP, especially at pH lower than 7.

The results were described in the publication :

J. Krasowska, M. Olasek, A. Bzowska, P.L. Clark, B. Wielgus-Kutrowska (2010) *„The comparison of aggregation and folding of enhanced green fluorescent protein (EGFP) by spectroscopic studies”* Spectroscopy 24, 343-348.

In the next paper:

J. Krasowska, K.G. Uriginov, P.L. Clark, A. Sienkiewicz, A. Bzowska, B. Wielgus – Kutrowska (2014) *„Spectroscopic properties of two single-cysteine mutants of EGFP: C48S-EGFP and C70S-EGFP”* Biomedical Spectroscopy and Imaging, accepted for publication,

we report the obtaining, stability and spectroscopic characterization of two single-cysteine mutants, C48S-EGFP and C70S-EGFP. Such mutants could be good, new tool for folding and dynamics studies by electron paramagnetic resonance (EPR) method, especially in the close vicinity of both cysteine residues, because of the possibility of labeling only one cysteine inside the protein.
Chromophore absorption, excitation and emission spectra for these mutants were similar to those of EGFP. Both single-cysteine mutants were more susceptible to aggregation than EGFP during expression in *E. coli*. C48S-EGFP exhibited similar resistance to chemical denaturation as EGFP. In contrast, C70S-EGFP was less resistant to denaturation in guanidinium hydrochloride and its folding was less efficient. Hence, C48S-EGFP seems to be a more suitable probe for EPR measurements than C70S-EGFP.

II. Other investigations using purine nucleoside phosphorylase which are not classified as a scientific achievement.

A. Study of the structure and mechanism of catalysis of the hexameric purine nucleoside phosphorylase from E. coli

Purine nucleoside phosphorylase from *Escherichia coli* differs in sequence, structure and substrate specificity from trimeric phosphorylases. It is hexamer (molecular mass 156 kDa) and belongs to the group of „high molecular mass” phosphorylases. It catalyzes not only the phosphorolysis of 6 - oxopurine nucleosides as „low molecular weight phosphorylases”, but also 6-amino-purine nucleosides.

In the first paper we have described the crystal structure of the ternary complex of hexameric purine nucleoside phosphorylase (PNP) from *Escherichia coli* with formycin A derivatives and phosphate or sulphate ions at 2.0 Å resolution. The hexamer is found as a trimer of unsymmetric dimers, which are formed by pairs of monomers with active sites in different conformations. The conformational difference comes from a flexible helix H8 (H8: a.a. 214-236), which is continuous in one conformer, and segmented in the other. For the continuous helix, the entry into the active site pocket is wide open, and the ligands are bound only loosely („open" or „loose binding" conformation). By segmentation of the helix the entry into the active site is partially closed, the pocket is narrowed and the ligands are bound more tightly („closed" or „tight binding" conformation). Furthermore, the side-chain of Arg217 is carried by the moving helix into the active site in tight binding conformation. This residue plays an important role in the proposed catalytic mechanism including the steps of:
1) formation of a hydrogen bond between the donor - Asp204 side chain and acceptor - nitrogen in the purine N^7 position
2) binding of the phosphate in the active site, what stabilizes the side chain of Arg24 and causes segmentation of H8 helix
3) conformational changes leading to shift of the side chain of Arg217 towards Asp204 to hydrogen bond distance

4) breaking of the hydrogen bond between Asp204 and the nitrogen of a purine base and the creation of bonds Asp204 - Arg217. The purine base with a proton attached becomes positively charged oxocarbonic ion with the features of transition state.

Two types of binding sites observed in the protein structure seems to confirm the results of the experiments performed in the solution in which the negative cooperation between the monomers in the dimer or the strong and weak binding sites are observed.

The results of these investigation are described in the paper:

G. Koellner, A. Bzowska, B. Wielgus-Kutrowska, M. Luić, T. Steiner, W. Saenger, J. Stępiński (2002) *„Open and Closed Conformation of the E. coli Purine Nucleoside Phosphorylase. Active Center and Implications for the Catalytic Mechanism”* J. Mol. Biol. 315, 351-371.

To confirm the proposed reaction mechanism we conducted additional experiments including directed mutagenesis, spectroscopic investigations of ligand binding and catalysis, and crystallographic studies.

So far, we have used PNP, which was a gift of Dr. George Koszałka (Wellcome Research Labs). To continue our investigations, we had to have access to the large amounts of protein.

We wanted to obtain PNP by overexpression in *Escherichia coli*. Such overexpression is described in the literature [Lee J., Filosa S., Bonvin J., Guyon S., Aponte R.A., Turnbull J.L. (2001) Protein Expr. Purif., 22,180-188]. We ask Dr. Turnbull to give us the plasmid of PNP. In collaboration with the group of prof. Marija Luić from Rudjer Bosković Institut in Croatia we have obtained the recombinant PNP from *Escherichia coli* and its mutants. This allowed us to continue studies of the mechanism of phosphorolysis by purine nucleoside phosphorylase from *Escherichia coli*.

Since now, the experimental evidence on the role of the chosen amino acid in catalysis has not been available. Therefore, the active site mutants Arg24Ala, Asp204Ala, Asp204Asn, Arg217Ala and Asp204Ala/Arg217Ala were prepared. The activity tests with natural substrates and 7-methylguanosine confirmed the earlier hypothesis, that catalysis involves protonation of the purine base at position N^7 by Asp204, which is triggered by Arg217. For the wild-type PNP and Arg24Ala mutant negative cooperation was observed in the case of phosphate binding.

The crystal structures of the wild type PNP in complex with phosphate and sulphate, and of the Arg24Ala mutant in complex with phosphate/sulphate were determined. Structure of wild-type PNP with phosphate complex, without the presence of ammonium sulfate, obtained with a resolution of 2.0 A, contains three hexamers of PNP in asymmetric unit. In contrast to the previous structure, for which the number of active sites in the closed and open conformations were equal, in this case each molecules have two active sites in the closed and four active sites in the open conformation. Crystallographic data showed that the previously observed [G. Koellner et al., (2002) J. Mol. Biol. 315, 351-371] protein conformational change leading to the placement of Arg217 nearby Asp204, important for catalysis, is due to binding of phosphate and its interaction with Arg24.

The results of these investigation are presented in the publication:

G. Mikleušević, Z. Stefanić, M. Narczyk, B. Wielgus-Kutrowska, A. Bzowska, M. Luić (2011) *„Validation of the catalytic mechanism of Escherichia coli purine nucleoside phosphorylase by structural and kinetic studies”* Biochimie 93, 1610-1622.

Next, the crystal structure of the ternary complex of this enzyme with a phosphate ion and formycin A was obtained.

The asymmetric unit were contained three different monomers, labeled A, B and C. The two types of dimers are formed: by the monomers A and C, and by monomer B and monomer B' received through symmetry operations. Two types of binding sites were identified - open (in the monomer B and C) and closed (in the monomer A). Monomer A bounds one molecule of phosphate. The structure reveals, in the active sites of monomers B and C, an unexpected and never before observed binding site for phosphate and exhibits a stoichiometry of two phosphate molecules per enzyme subunit

The PNP titration with phosphate was performed. The additional weakly bound phosphate site visible in crystallography, giving a stoichiometry of two molecules of phosphate per monomer was not confirmed by solution studies.

The results of these investigation are included in the paper:

Z. Štefanić, M. Narczyk, G. Mikleušević, B. Wielgus-Kutrowska, A. Bzowska, M. Luić (2012) ***"New phosphate binding sites in the crystal structure of Escherichia coli purine nucleoside phosphorylase complexed with phosphate and formycin A"*** **FEBS Letters 586, 967-971.**

Next, as a review we have summarized the results of the above-mentioned, earlier work concerning the determination of the mechanism of catalysis in the presence of *E. coli* PNP. We have discussed a unique stoichiometry of binding of phosphate (two molecules per monomer of PNP), which can result from a very high concentration of phosphate or from a long period of crystallization of the protein. We have also discussed a negative cooperation between the subunits in the oligomeric enzyme. To unambiguously determine the stoichiometry of binding of the ligands in solution, particularly phosphate and describe the phenomenon of cooperation between subunits PNP and/or active sites non-identity the additional tests are needed.

Summary of knowledge of on *E. coli* PNP phosphorolysis are collected in the review:

Z. Štefanić, G. Mikleušević, M. Narczyk, B. Wielgus-Kutrowska, A. Bzowska, M. Luić (2013) ***„Still a Long Way to Fully Understanding the Molecular Mechanism of Escherichia coli Purine Nucleoside Phosphorylase"*** **Croat. Chem. Acta 86, 117–127,**

The purine nucleoside phosphorylase from *E. coli* is a hexamer , which can be considered as a trimer of dimers. It appears that the dimer is the smallest unit which can catalyze the phosphorolysis in accordance with the previously proposed mechanism. To investigate the phenomenon of communication between the subunits and find the answer for question why the biologically active form of the enzyme exists as a hexamer we tried to obtain dimers of PNP from *E. coli*. In the first step by molecular modeling methods we have checked which mutations on the contact surface between the dimers may cause the destabilization of the hexamer. Next, we have constructed mutants of PNP but we have not obtained stable dimers. The molecular modeling showed that such dimer does not have the proper three-dimensional structure. Circular dichroism measurements showed changes in the secondary structure of the protein after the mutation. Analytical centrifugation experiments demonstrated that the mutants do not form stable dimers, but rather dissociate into monomers. The

catalytic activity of the mutants is negligible, six orders of magnitude smaller than that of the wild-type protein.

Description of our work we have presented in the publication :

B. Bertoša, G. Mikleušević, B. Wielgus-Kutrowska, M. Narczyk, M. Hajnić, I. Leščić Ašler, S. Tomić, M. Luić, A. Bzowska (2014) *„Homooligomerization is needed for stability: molecular modeling and solution study of E. coli purine nucleoside phosphorylase”*, FEBS J. 281, 1717–1930.

B. Study of the structure of trimeric purine nucleoside phosphorylases from calf spleen and Cellulomonas sp and mechanism of catalysis.

Most of the studies on trimeric phosphorylases is presented as a scientific achievement. The set of publications, which have not been selected for this purpose contained a few communications on the interaction of *Cellulomonas* and calf spleen PNP with different ligands.

We have described the phosphorolysis of a typical substrate - inosine (Ino) and non-typical - 7-methylguanosine (m^7Guo) and phosphate, catalyzed by the of PNP from *Cellulomonas sp.* We have analyzed the kinetics of the synthesis of guanosine (Guo) and product inhibition of these reaction.

The results are published in the communication :

B. Wielgus-Kutrowska, A. Bzowska (2005) *„Kinetic properties of Cellulomonas sp. purine nucleoside phosphorylase with typical and non-typical substrates: implications for the reaction mechanism”* Nucleosides, Nucleotides and Nucleic Acids 24, 471-476.

We have measured the dissociation constants and binding stoichiometry for the interaction of the trimeric PNP (from calf spleen and *Cellulomonas sp.*) with the ground state analogues (substrates and inhibitors).

The results of these studies are included in the communication :

B. Wielgus-Kutrowska, A. Holý, J. Frank, G. Koellner, A. Bzowska (2003) *„Interactions of trimeric purine nucleoside phosphorylases with ground state analogues - calorimetric and fluorimetric studies”* Nucleosides, Nucleotides and Nucleic Acids 22, 1695-1698.

We have analyzed the formation of 2-amino-9-[2-(phosphonomethoxy)ethyl]-6-sulfanylpurine with the trimeric purine nucleoside phosphorylase from *Cellulomonas sp* complex.

The results of investigations are presented in the communication:

J.M. Antosiewicz, B. Wielgus-Kutrowska, M. Długosz, A. Holý, A. Bzowska (2007) *„Kinetics of binding of multisubstrate analogue inhibitor (2-amino-9-[2-(phosphonomethoxy)ethyl]-6-sulfanylpurine) with trimeric purine nucleoside phosphorylase”* Nucleosides, Nucleotides and Nucleic Acids 26, 969-74.

We have described the preliminary results of PCR amplification of the phosphorylase from a bovine spleen cDNA library, cloning, overexpression of PNP, enzymatic activity and interactions with typical ligands.

Preliminary results of these studies are collected in the communication:

K. Stepniak, A. Girstun, B. Wielgus-Kutrowska, K. Staron, A. Bzowska (2007) *„Cloning, expression, purification, and some properties of calf purine nucleoside phosphorylase”* Nucleosides Nucleotides Nucleic Acids 26, 855-859.

We have determined entropy/entalphy contribution to the interaction of purine nucleoside phosphorylase (PNP) from calf spleen with multisubstrate inhibitors: DFPP-DG, DFPP-G and (S)-PMP-DAP.

Preliminary results of these studies are included in the communication:

K Breer, B. Wielgus-Kutrowska, M. Hashimoto, S. Hikishima, T. Yokomatsu, R.H. Szczepanowski, M. Bochtler, A. Girstun, K. Staroń, A. Bzowska (2008) *„Thermodynamic studies of interactions of calf spleen PNP with acyclic phosphonate inhibitors”* Nucleic Acids Symp. Ser. 52, 663-664.

We performed tests of Dynafit program for analyzing the data describing the interactions between enzyme and ligand.

The results of these studies are presented in the communication:

J.M. Antosiewicz, K. Breer, A. Bzowska, B. Wielgus-Kutrowska (2008) *„On the analysis of fluorimetric titration curves of purine nucleoside phosphorylase”* Nucleic Acids Symp Ser. 52, 671-672.

We have tested the change of oligomeric state of PNP during controlled deactivation of enzyme.

The results of these studies are presented in the communication:

B.Wielgus-Kutrowska, A. Modrak-Wojcik,;A. Dyzma, M. Zolkiewski, A. Bzowska (2013) *„Inactivation of trimeric purine nucleoside phosphorylase: analytical ultracentrifugation studies”* Eur. Biophys. J. 42 Suppl. S72-S72.

C. Investigation of 8-apurine derivatives properties from the point of view of their interaction with „low-“ and „high molecular mass” purine nucleosides phosphorylases.

The possibility of obtaining of recombinant PNP in any amount made possible to cooperate with the group of Prof. Jacek Wierzchowski, who checked the interactions of 8-azapurines derivatives with enzymes from mammalian and bacterial sources.

The recombinant calf PNP catalyzes ribosylation of 2,6-diamino-8-azapurine in a phosphate-free medium, with ribose-1-phosphate as ribose donor, but the ribosylation site is predominantly N^7 and N^8, with the proportion of N^8/N^7 ribosylated products markedly dependent on the reaction conditions. Both products are fluorescent. Application of the *E. coli* PNP gave a mixture of N^8 and N^9-substituted ribosides. Fluorescence of the ribosylated 2,6-diamino-8-azapurine has been briefly characterized. The highest quantum yield, ~0.9, was obtained for N^9-β-d-riboside (λ_{max} 365 nm), while for N^8-β-d-riboside the fluorescence quantum yield was found to be close to 0.4 at ~430 nm. Ribosylation of 8-azaguanine

with calf PNP as a catalyst goes exclusively to N^9. By contrast, the *E. coli* PNP ribosylates 8-azaGua predominantly at N^9, with minor, but highly fluorescent products ribosylated at N^8/N^7.

The measurement results are presented in the publication:

A. Stachelska-Wierzchowska, J. Wierzchowski, B. Wielgus-Kutrowska, G. Mikleušević (2013) *„Enzymatic Synthesis of Highly Fluorescent 8-Azapurine Ribosides Using a Purine Nucleoside Phosphorylase Reverse Reaction: Variable Ribosylation Sites”* Molecules 18, 12587-12598.

Two non-typical nucleosides, 7-β-D-ribosyl-2,6-diamino-8-azapurine and 8-β-D-ribosyl-2,6-diamino-8-azapurine, have been found to exhibit moderately good, and selective, substrate properties toward calf and bacterial (*Escherichia coli*) forms of purine nucleoside phosphorylase (PNP). Both compounds and the reaction products are fluorescent.

The results of fluorescence measurements are presented in the publication:

J. Wierzchowski, A. Stachelska-Wierzchowska, B. Wielgus-Kutrowska, G. Mikleuševic (2014) *„Two fluorogenic substrates for purine nucleoside phosphorylase, selective for mammalian and bacterial forms of the enzyme”* Anal. Biochem. 446, 25–27.

6.3. Scientific research before obtaining a PhD degree

Main results of research before obtaining a PhD degree are:

III. Description of the spectral properties of 8-aza-purine nucleosides and their nucleosides and characterization of their interactions with enzymes from calf spleen and Escherichia coli.

The fluorescence emission properties of the neutral and ionic forms in aqueous medium of the azapurine nucleosides, 8-azaadenosine (8-azaAdo), 8-azainosine (8-azaIno), 8-azaguanosine (8-azaGuo), and their aglycons were studied. The fluorescence of 8-azaGuo and 8-azaIno at pH 7 originates from their anionic form, whereas 8-azaAdo is a strong emitter as the neutral form. The corresponding 8-azapurines are weakly fluorescent in aqueous medium, with the exception of 8-azaguanine (8-azaG).

- 8-azaG and 8-azaHx are substrates for calf spleen PNP in the 8-azapurine nucleosides synthesis reaction. In the case of *E. coli* PNP additional substrate is a 8-azaA, what is consistent with the fact that adenosine is a substrate of the phosphorolysis catalyzed by *E. coli* PNP, but not for phosphorylase from calf spleen.
- The 8-azapurine nucleosides are reasonably good inhibitors of inosine (Ino) and guanosine (Guo) phosphorolysis catalyzed by *E. coli* PNP. The most effective is 8-azaIno (K_i approx. 20 μM). 8-azaIno is the only one inhibitor of phosphorolysis catalyzed by the calf spleen enzyme (K_i approx. 40 μM).

The results of measurements were published in:

J. Wierzchowski, B. Wielgus-Kutrowska, D. Shugar (1996) *„Fluorescence emission of 8-azapurines and their nucleosides, and application to the kinetic reverse synthetic reaction of purine nucleoside phosphorylase”* Biochim. Biophys. Acta 1290, 9-17.

IV. Characterization of nicotinamide riboside as a substrate and inhibitor of purine nucleoside phosphorylases and a proposal of a model of nicotinamide riboside binding at the binding sites of calf spleen and E. coli PNP.

Nicotinamide riboside (NIR) differs from the natural substrates - it is a positively charged pyridine derivative, whereas the natural substrates are derivatives of purines. I have measured the kinetics of phosphorolysis of NIR and, for comparison, inosine.
NIR has been confirmed as an unusual substrate for purine-nucleoside phosphorylase (PNP) from a mammalian source (calf spleen) and from bacteria (*Escherichia coli)*. It binds in the same region of the active site as natural substrates. The K_M values at pH 7, 1.48 mM and 0.62 mM,, were 1-2 orders of magnitude higher than for the inosine, but the V_{max} values were comparable, 96% and 35% that for Ino, respectively for calf spleen and *Escherichia coli*.
NIR was a weak competitive inhibitor of inosine phosphorolysis catalyzed by both enzymes. Phosphorolysis of the fluorescent positively charged substrate 7-methylguanosine was also inhibited in a competitive manner by both Ino and NIR.
The pH dependence of the kinetic constants for the phosphorolysis of NIR and Ino, was extensively investigated. In the alkaline pH range, NIR exhibited abnormally high substrate activity in comparison with the reduced reaction rates of both enzymes towards Ino. The decrease in maximum speed of inosine phosphorolysis is likely due to either unfavorable ionic forms of Ino, or because of changes in the ionic state of amino acid residues present in the active site of PNP.

On the basis of the kinetic studies and literature data we have suggested the binding model of NIR in the active site of calf spleen PNP. Most likely NIR forms a hydrogen bonds with Asn243 and Glu201, caused by the presence in its structure groups simulating the Ino and Guo fragments (C^6=O, and N^1-H).

The results are summarized in the publication:

B. Wielgus-Kutrowska, E. Kulikowska, J. Wierzchowski,A. Bzowska, D. Shugar (1997) *„Nicotinamide riboside - an unusual, non-typical substrate of purified purine nucleoside phosphorylases”* Eur. J. Biochem. 243, 408-414.

V. Determination of the three-dimensional structure, the characteristics of the interaction with substrates and inhibitors and a proposal for the mechanism of binding and phosphorolysis of nucleosides by Cellulomonas PNP.

The commercially available PNP from *Cellulomonas sp*. was significantly contaminated. The pure PNP was about 20% of the sample. We designed a method of PNP purification by ion exchange chromatography technique. Binary complex of the purified enzyme with phosphate was crystallized with the enzymatically active trimer in the asymmetric unit. The structure was solved (resolution of 2.2 Å) by molecular replacement with calf spleen PNP as a model. A ternary complex of the enzyme with phosphate and 8-iodoguanine was obtained by soaking crystal of the binary orthophosphate complex with very weak substrate 8-iodoguanosine (it scatters the X-ray with the resolution 2.4 Å). The three-dimensional structure of PNP shown preservation of the amino acids important for catalysis in the active site in comparison to enzymes from mammalian sources –calf spleen and human erythrocytes.

We have analyzed the kinetics of the interaction of PNP with inorganic phosphate and nucleoside. We have found that, despite of the similarities in the amino acid composition of binding sites, specificity of *Cellulomonas* PNP differs from that of calf spleen PNP and human erythrocytes because:
a) adenosine is an inhibitor of PNP from *Cellulomonas sp*, while it does not bind (or binds very weakly) to the enzymes from mammalian sources, and it is a substrate for the *E. coli* PNP,
b) inhibitors which bind to the mammalian phosphorylase only or that, which bind to *E. coli* PNP only, are the inhibitors of *Cellulomonas* PNP.

The crystallographic structure of *Cellulomonas* PNP and inhibitory properties of adenosine and formycin A allowed to propose a mechanism of phosphorolysis in which Glu204 (Glu201 in the calf, and the human enzyme) plays a key role, while Asn246 (Asn243 in mammalian enzymes) provides rather the bonding of a 6-oxopurines than catalysis. This mechanism is consistent with the properties of phosphorylases from mammalian sources and explains the substrate properties of the NIR and N^7-purine nucleosides as a substrates of trimeric phosphorylases. It is also consistent with their specificity to the 6-oxopurine nucleosides and kinetic properties of the PNP mutants Asn243/Ala and Glu201/Ala from human erythrocytes.

The results of the enzyme from *Cellulomonas sp.* investigation were included in the following communications and publications:

J. Tebbe, B. Wielgus-Kutrowska, W. Schröder, M. Luić, D. Shugar, W. Saenger, G. Koellner, A. Bzowska (1996) *„Purine nucleoside phosphorylase (PNP) from Cellulomonas sp.,a third class of PNP different from both „low-molecular weight" mammalian and „high-molecular weight" bacterial PNPs"* Miami, Nature Biotechnology, Short Reports, 8, 90,

B. Wielgus-Kutrowska, J. Tebbe, W. Schröder, M. Luić, D. Shugar, W. Saenger, G. Koellner, A. Bzowska (1998) *„Cellulomonas sp. Purine Nucleoside Phosphorylase (PNP): Comparison with Human and E. coli Enzymes"* Adv. Exp. Med. Biol. 431, 259-264,

A. Bzowska, J. Tebbe, M. Luić, B. Wielgus-Kutrowska, W. Schröder, D. Shugar, W. Saenger, G. Koellner (1998) *„Crystallization and preliminary studies of purine nucleoside phosphorylase from Cellulomonas sp."* Acta Cryst. D54, 1061-1063,

J. Tebbe, A. Bzowska, B. Wielgus-Kutrowska, W. Schröder, Z. Kazimierczuk, D. Shugar, W. Saenger, G. Koellner (1999) *„Crystal structure of the Purine Nucleoside Phosphorylase (PNP) from Cellulomonas sp. and its implication for the mechanism of trimeric PNPs"* J. Mol. Biol. 294, 1239-1255,

B. Wielgus-Kutrowska, J. Tebbe, J. Wierzchowski, D. Shugar, W. Saenger, G. Koellner, A. Bzowska (1999) *„Binding of substrates by purine nucleoside phosphorylase (PNP) from Cellulomonas sp. – kinetic and spectrofluorimetric studies"* Nucleosides, Nucleotides and Nucleic Acids. 18, 871-872.

Emission spectroscopy methods was employed to the investigation of interaction of substrates and products of phosphorolysis conducted by PNP from *Cellulomonas sp*.

Purine bases, R1P, and phosphate enhance the intrinsic fluorescence of *Cellulomonas* PNP – formation of a binary complexes induces conformational changes of the protein in the microenvironment of

tryptophan residue(s). The effect due to guanine (Gua) binding is much higher than those caused by other ligands, suggesting that the enzyme preferentially binds a fluorescent, rare tautomeric anionic form of Gua. Guanosine and inosine at 100 μM concentration show little and no effect, respectively, on enzyme intrinsic fluorescence, but the investigations of their protective effect against thermal inactivation of the enzyme show that they form weak binary complexes with PNP. Binding of Gua, Hx and R1P to the trimeric enzyme is described by one dissociation constant, K_d=0.46 μM for Gua, 3.0 μM for Hx, and 60 μM for R1P. The binding stoichiometry for Gua (and probably Hx) is three ligand molecules per enzyme trimer. Effects of phosphate on the enzyme intrinsic fluorescence are due not only to binding, but also to an increase in ionic strength, as shown by titration with KCl. When corrected for effects of ionic strength, titration data are most consistent with one dissociation constant for phosphate, K_d=270 μM, but existence of a very weak binding site with K_d>50 mM could not be unequivocally ruled out. Binding of Gua to the PNP/phosphate binary complex is weaker (K_d=1.7 μM) than to the free enzyme (K_d=0.46 μM), suggesting that phosphate helps release the purine base during the phosphorolysis. The results indicate that nonlinear kinetic plots of initial velocity, typical for PNPs, including *Cellulomonas* PNP, are not, as generally assumed, due to cooperative interaction between monomers forming the trimer, but to a more complex kinetic mechanism.

Result of these studies allowed to write the following publication, published after obtaining a PhD degree:

B. Wielgus-Kutrowska, A. Bzowska, J. Tebbe, G. Koellner, D. Shugar (2002) *„Purine Nucleoside Phosphorylase (PNP) from Cellulomonas sp.: physicochemical properties and binding of substrates determined by ligand – dependent enhancement of enzyme intrinsic fluorescence, and by protective effects of ligands on thermal inactivation of the enzyme”* Biochim. Biophys. Acta 1597, 320-334.

VI. Study of the interactions of the trimeric and hexameric PNP with synthesized 2-chloro-6-aryloxy-and 2-chloro-6-alkoxyarylpurines

We have synthesized a series of 2-chloro-6-aryloxy- and 2-chloro-6-alkoxyarylpurines and we have determined their kinetic properties in the purine nucleoside phosphorylase (PNP) system. All compounds showed inhibitory activity (IC50 in the range 0.5-76 μM) vs. hexameric PNP from *E. coli*. By contrast, no inhibition vs. trimeric *Cellulomonas* PNP was detected.

The results of investigations were published in the communication:

A. Bzowska, L. Magnowska, B. Wielgus-Kutrowska, Z. Kazimierczuk (1999) *„Synthesis of 2-chloro-6-aryloxy- and 2-chloro-6-alkoxyarylpurines and their properties in purine nucleoside phosphorylase (PNP) system”* Nucleosides, Nucleotides and Nucleic Acids. 18, 873-874.