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VAZEBNÉ VLASTNOSTI ENZYMŮ PURINNUKLEOSIDFOSFORYLASA A THYMIDINFOSFORYLASA

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LIGAND BINDING PROPERTIES OF PURINE NUCLEOSIDE PHOSPHORYLASE AND THYMIDINE PHOSPHORYLASE

Kateřina Strašilová **2006**

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Katerina Strasilova

ABBREVIATIONS

Ade	adenine
Ado	adenosine
dAdo	deoxyadenosine
dGuo	deoxyguanosine
dIno	deoxyinosine
dR-1-P	deoxyribose-1-phosphate
F-Ado	2-fluoroadenosine
Fc	flow cell
F-dAdo	2-fluoro-2´-deoxyadenosine
Gua	guanine
Guo	guanosine
IFC	integrated μ-fluidic cartridge
Ino	inosine
m^7Ado	7-methyladenosine
m^7Guo	7-methylguanosine
MESG	6-thio analogue of m^7Guo
NR^+	nikotinamide ribose
PNP	purine nucleoside phosphorylase
R-1-P	ribose-1-phosphate
SPR	surface plasmon resonance
TP	thymidine phosphorylase
Xao	xanthosine

INTRODUCTION

Natural nucleosides and especially their modified analogues either directly or as intermediates are of great pharmaceutical interest in the development of antiviral and antitumour agents. Modified nucleosides are classical antiviral drugs, commonly used in the treatment of herpes virus and HIV infections. They usually act as chain terminators in viral nucleic acid replication, although in some cases other mechanisms have been suggested, such as inhibition of RNA capping or hypermutagenesis of viral nucleic acids [15]

Nucleosides bearing different modifications either on the ribofuranosyl moiety or on the sugar-linked heterocyclic nitrogen bases can be prepared by chemical synthesis through multistage processes requiring protection and deprotection of labile groups. These synthetic approaches are often accompanied by low overall yields and by formation of regio- and stereo- isomers. The biocatalyst technology has many advantages over synthetic approaches in producing structurally complicated fine chemicals like most pharmaceuticals.

Biocatalysis usually replaces multi-step chemical processes and minimize energy consumption and disposal of waste, and subsequently the cost of the final product.
It also allows production of novel bioorganic molecules, which can't be synthesized.
This work is related to the research project „Biocatalysts for producing pharmaceutical nucleoside analogues", where the biocatalysis utilising the enzyme Purine nucleoside phosphorylase (PNP) is used for producing purine nucleoside analogues. This study is focused on analysing of these two enzymes - Purine nucleoside phosphorylase and Thymidine phosphorylase and especially on analysing their binding properties using a phenomenon of surface plasmon resonance in Biacore X instrument.

The whole project has several participants; they are University of Kuopio, University of Oulu and University of Helsinki. This study was performed in the group of University of Kuopio, which is developing biocatalysts for the synthesis of nucleosides and their analogues to be studied as potential drugs or as raw materials for such.

1. REVIEW OF LITERATURE

1.1 SURFACE PLASMON RESONANCE ANALYSIS

Surface plasmon resonance (SPR) is a non-invasive optical measuring technique which measures the mass concentration of biomolecules on the sensor surface. SPR causes a reduction in the reflected light intensity at a specific combination of angle and wavelength *(generating a refractive index dependent SPR signal).* The technique does not require any labelling of the interacting components. The response is essentially independent of the nature of the biomolecule, so that all steps in an interaction analysis are followed with the same criterion.

Biacore X is a semi-automated system for analyzing interactions between biomolecules and their ligands using a phenomenon of surface plasmon resonance. In Biacore terminology, a molecule, which is immobilized to the surface of the sensor chip, is called *ligand* and the other one, which is free in the solution flowing over the surface, is called *analyte*.

Biacore X provides high sensitivity in small consumption of samples and allows measuring wide range of biomolecules - from purified molecules to crude samples, like lipid vesicles, viruses, bacteria and eukaryotic cells.

Usual types of analyses are specificity analysis, multiple binding analysis, concentration analysis, affinity and kinetic analysis, studies of enzymatic processes and studies of structure-function relationships.

Specificity analysis answers the question if the molecule is specific for its target.

Multiple binding analysis investigates how different components in a complex bind to each other.

Concentration analysis gives the answer of how much analyte is there in a sample.

Affinity and kinetic analyses deal with strength of binding between ligand and analyte and with velocity of association and dissociation of the complex.

Studies of enzymatic processes involve proteolysis, phosphorylation, nucleic acid synthesis, ligation and cleavage.

Studies of structure-function relationship elucidate functional changes according to structural modifications and variations in a molecule.

Biacore X technology consists of three main parts. They are microfluidics, the sensor chip and the SPR detection.

Microfluidics

The microfluidic system consists of a series of channels and valves in a plastic block, the Integrated μ-Fluidic Cartridge (IFC). The flow cells are formed by pressing the sensor chip against a set of open channels on the surface of the IFC, so that the chip can easily be exchanged. Delivery of sample and buffer to the flow cells is precisely controlled by the pump system and the valves in the IFC. In this way, a continuous flow of liquid is maintained over the sensor surface throughout the analysis, switching between buffer and sample with minimum disturbance or dispersion of the sample boundary. This precision in sample delivery is very important for reproducibility of assay procedures, and also provides the control conditions necessary for interpreting kinetic data obtained from the interaction studies.

There are two flow cells in Biacore X and they can be used individually or in the series. When used individually, two different ligands can be immobilized. But when used in series, one of them serves for immobilization and the other serves as a reference. The in-line reference subtraction runs automatically. The microfluidic system is miniaturized and designed to use as little as 5μl of sample, while volume of the cell is 0.06μl. The whole system is in a temperature controlled environment, liquid handling is automated and integrated. Elimination of air/liquid interfaces minimizes sample dispersion and protein denaturation.

The Biacore Sensor chip

The surface of the sensor chip serves to immobilize of the ligand and it also creates one wall of the flow cell. The chip provides the physical conditions necessary to generate the SPR signal and all the interactions which are studied take place on its surface. Immobilization of the ligand to the surface of the chip can be done by three different

approaches; direct coupling to the surface creating the covalent bond between molecules, non-covalent capture via a suitable additive molecule already coupled to the surface and hydrophobic adsorption with molecule of interest or hydrophobic carrier of lipid character.

The sensor chip consists of glass support covered with a thin layer of gold - this creates the conditions to generate the SPR response - and on the gold layer there is some coating providing an environment to attach the ligand. The coating varies between different types of chips and also uncoated chip is available when users need to prepare their own surface for special applications. The sensor chip itself is mounted on a plastic carrier placed in the plastic sheath to facilitate handling.

Biacore provides a range of sensor chips; therefore the choice depends on the nature of molecule to be coupled and the requirements for the analysis. In this study we used the Sensor chip CM5, the most versatile chip available. It is suitable for ligand fishing and for high capacity capture; it supports a wide range of immobilization levels, attaches proteins, nucleic acids, carbohydrates or small molecules; carboxyl groups on the surface can couple wide range of other active groups (-NH_2, -SH, -CHO, -OH or -COOH)

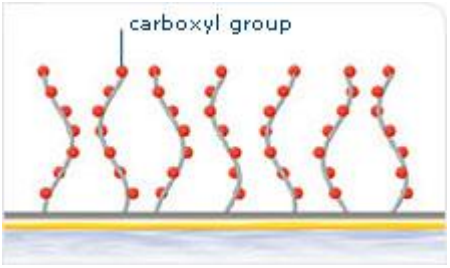


Fig.1 Simple illustration of the sensor chip surface (www.biacore.com)

A dextran matrix is used as the coating here. The dextran belongs to most common coatings of Biacore chips, but they are available with several the different properties. The dextran matrix is hydrophilic, flexible with low non-specific binding and high binding capacity. The matrix in CM5 is a carboxymethylated dextran covalently attached to the gold layer. The protein molecules to be studies are covalently coupled to the sensor surface via amine, thiol, aldehyde, hydroxy or carboxyl groups. High surface stability allows repeated analysis on the same surface. Analyte interactions with protein

are monitored by injecting samples over the prepared surface of a sensor chip. Between injections, the surface is regenerated by selective dissociation of the interaction partners. Regeneration solutions ensure complete dissociation, without affecting the binding characteristics of the immobilized partner.
The surface concentration of the immobilized interaction partner varies according to the type of analysis. Generally concentration and specificity assays require a high surface concentration, while lower concentrations are preferable for kinetic analysis.

Closer view to each of the components

The gold layer

The gold layer is uniform and app. 50 nm thick. It provides good conditions for surface plasmon resonance. It gives a well-defined reflectance minimum at easily handled visible light wavelengths, and it is also suitable to covalent bonding of dextran matrix layers at the same time as the metal is largely inert in physiological buffer conditions.

The dextran matrix

A matrix of carboxymethylated dextran is covalently attached to the gold surface. This layer is approximately 100 nm thick. The dextran matrix close to the surface is equivalent in concentration to an aqueous solution of about 2% dextran and imparts these important properties:

- It provides a hydrophilic environment favourable to most solution
- It provides defined chemical basis to covalent coupling using a variety of chemical reactions
- Flexibility, which allows relatively free movement of attached ligands
- The negatively charged carboxyl groups allow electrostatic concentration of positively charged molecules from solution that enable effective immobilization
- It increases the surface capacity for immobilization as well as the region where interaction occurs

Optical system - SPR

Biacore system uses surface plasmon resonance (SPR) to monitor biomolecular binding events in real time without the use of labels. The SPR is a phenomenon that occurs in thin conducting films at an interface between media of different refractive index. In Biacore systems, the media are the glass of the sensor chip and the sample solution; the conducting film is a thin layer of gold on the sensor chip surface.

Molecules binding to the sensor surface cause changes in the refractive index close to the surface which are detected as changes in the SPR signal (expressed in resonance units, RU where one RU is equivalent to one picogram per square millimeter on the sensor surface). In general, the refractive index change for a given change of mass concentration at the surface layer is practically the same for all proteins and peptides, and is similar for glycoproteins, lipids and nucleic acids.

Detection unit consists of an optical unit which includes light source and detector, the opto-interface and the removable sensor chip. Forming of each of the components is as follows. The glass side of the sensor chip is pressed into the contact with a glass prism in the optical unit. A silicone opto-interface is between the sensor chip and the prism, (matched in refractive index to the glass, ensures good optical coupling between the prism and the removable sensor chip.)

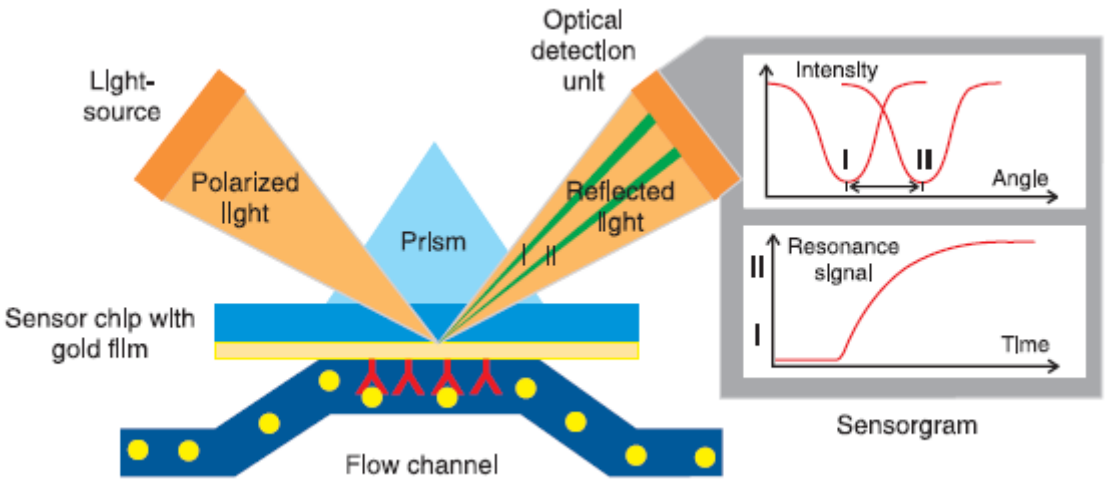


Fig. 2 The scheme of detecting unit and SPR principle – BiacoreX-Handbook (www.biacore.com)

Light is directed at, and reflected from, the side of the surface not in contact with sample. SPR causes a reduction in the reflected light intensity at a specific combination of angle and wavelength. Light from a near-infrared light-emitting diode (LED) is focused through the prism on to the sensor surface in a wedge-shaped beam, giving a fixed range of incident light angles. Light reflected from the sensor chip is monitored by a linear array of light-sensitive diodes covering the range of incident light angles. The diodes are spaced with a resolution corresponding to approximately 0.1º, and computer interpolation algorithms determine the angle of minimum reflection (the SPR angle) to a high accuracy.

Under conditions of total internal reflection, light incident on the reflecting interface leaks an electric field intensity called an *evanescent wave field* across the interface into the medium of lower refractive index, without actually losing net energy. The amplitude of the evanescent field wave decreases exponentially with distance from the surface, and the effective penetration depth in terms of sensitivity to refractive index is about 20% of the wavelength of the incident light.

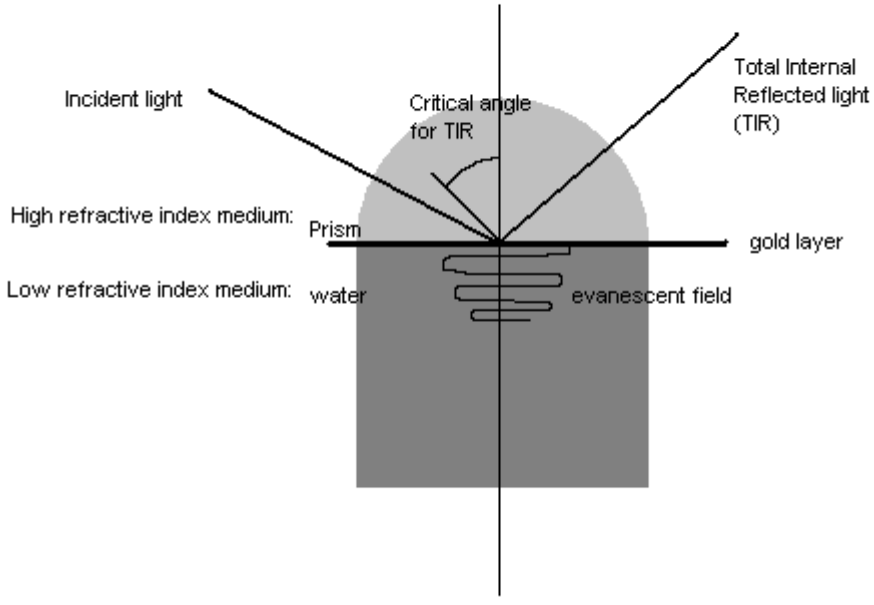


Fig. 3 Total Internal Reflection and SPR - Biacore basics; Introduction to SPR Analysis (edition December 2003)

At a certain combination of angle of incidence and energy (wavelength), the incident light excites plasmons (electron charge density waves) in the gold film. As a result, a characteristic absorption of energy via the evanescent wave field occurs and SPR is seen as a drop in the intensity of the reflected light. Because the evanescent wave field penetrates the solution, conditions for this resonance effect are very sensitive to the refractive index of the solution within the effective penetration depth of the evanescent field. Changes in solute concentration at the surface of the sensor chip cause changes in the SPR conditions. The penetration depth of the evanescent wave determines the thickness of the solution layer where refractive index changes are monitored: only refractive index changes close to the surface affect the SPR signal.

SPR arises in principle in any thin conducting film under the conditions described, although the wavelength at which resonance occurs and the shape of the energy absorption profile differ with different conducting materials. Gold is used in Biacore sensor chips because it combines favorable SPR characteristics with stability and a high level of inertness in biomolecular interaction contexts. Biomolecular binding events cause further changes in the refractive index and the SPR signal. Biacore X precisely controls every critical step to detect the small changes between a baseline signal and the signal generated when a second or third molecule interacts.

1.2. PURINE NUCLEOSIDE PHOSPHORYLASE

Purine nucleoside phosphorylase (PNP) is an enzyme, which catalyzes the cleavage of the glycosidic bond between oxygen and nitrogen in both purine ribonucleosides and deoxyribonucleosides. The presence of inorganic orthophosphate is needed as a second substrate. This reaction is for the natural substrates reversible.

β-purine nucleoside + P_i ↔ *purine base* + *α-D-pentose-1-phosphate*

Under in vivo conditions phosphorolysis is highly favoured over synthesis, due to coupling with two additional enzymatic reactions, oxidation and phosphoribosylation of the liberated purine bases by xanthine oxidase (Xox) and hypoxanthine-guanine phosphoribosyltransferase (HGPRT), respectively. The role in purine metabolism is in the salvage pathway. PNP enables the cells to utilize purine bases from metabolized purine ribo- and deoxyribonucleosides to synthetize purine nucleotides. [1]

PNP belongs to a class of N-ribohydrolases and transferases, where the transition states share ribosyl oxocarbenium-ion character, in which cleavage of C-N glycosidic bond occurs by an S_N1-like mechanism.

DISTRIBUTION IN NATURE

Various PNPases are widely distributed in the nature. PNP is a ubiquitous enzyme distributed in number of tissues and cells in mammals, birds, fish, yeast and several species of bacteria. [2] However, the enzyme activity differs both in each organism and each tissue or cell type. The highest activities in humans are found in kidneys, peripheral lymphocytes as well as in red cells or in granulocytes. According to volume, red cells are the richest source of PNP. On the other hand, erythrocyte PNP levels in mice are considerably lower. The highest activity of PNP in mice is in the proximal small intestine. [1]

ROLE IN METABOLISM

In intact cells, PNP functions in direction of phosphorolysis, leading to degradation of purine nucleosides via coupling with guanase and Xox. It was found, that histochemical localization of PNP in rat and human liver cells is similar to that of Xox, which converts Hx to urate via xanthine. [1] PNP functions principally in the direction of breakdown of guanine or hypoxanthine ribo- and deoxyribonucleosides. Adenosine and deoxyadenosine have, in addition to PNP, a specific kinase, which is unreactive with nucleosides of guanine or hypoxanthine. Also, the enzyme, adenosine deaminase, is found in a number of mammalian tissues. This enzyme catalyzes the deamination of adenosine/deoxyadenosine to inosine/deoxyinosine, which are latter substrates for PNP.
PNP can also be a salvaging enzyme for ribose and deoxyribose moieties of nucleosides. α-D-ribose-1-phosphate (R-1-P) can be converted by phosphoribomutase to ribose-5-phosphate, a precursor of 5-phosphoribosyl-1-pyrophosphate (PRPP), a co-substrate of HGPRT.[1, 2]

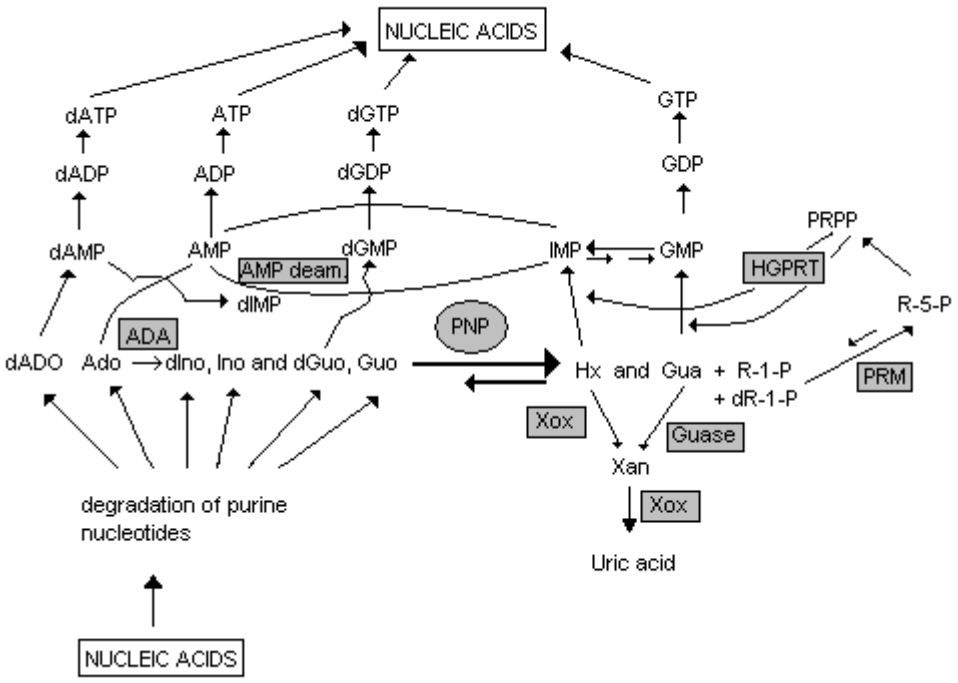


Fig. 4 Metabolic scheme; Bzowska et al. (2000) [1]

ROLE IN CHEMOTHERAPY

PNP can play a role in chemotherapy in these ways:

- catalyzing the breakdown of nucleosides containing purine analogs, which may be of anticancer or antiviral activity
- promoting the incorporation of purine/pyrimidine analogues into the nucleotides, nucleic acids by increasing intracellular pools of R-1-P or dR-1-P [2]

On the other hand, PNP deficiency is an inherited disease of purine metabolism, leading to T-cell immune deficiency, whereas B-cell function remains normal. Thus potent inhibitors of human PNP are potential selective immunosuppressive agents.

Moreover, many purine nucleoside analogues with potential chemotherapeutic activity are inactivated by cellular PNP before reaching their target cells. Therefore potent selective PNP inhibitors are considered useful to enhance chemotherapy, e.g. with 2′3′-dideoxyadenosine (with anti-HIV activity) which is a substrate for human PNP, or 2-chloro-2′-deoxyadenosine (anti-hairy-cell leukemia drug). The latter can not be applied orally since it is inactivated by PNP of enteric bacteria. [5]

MOLECULAR PROPERTIES

According to protein structure, there are two main classes of, which have been characterized. The so called "low molecular mass" phosphorylases are trimeric enzymes (Mr about 90 kDa) specific for 6-oxopurine nucleosides, often referred to "Ino-Guo phosphorylases" and found mainly, but not exclusively, in mammals (e.g. human, calf...). The second group, so-called "high molecular mass" phosphorylases (Mr in range 110-160kDa) are hexameric and found in microorganisms. Their specificity is broader; both 6-oxo and 6-aminopurine nucleosides serve as a substrate. This class includes the enzymes from *E. coli*, *Salmonella typhimurium*, *Klebsiella* and *Sulfolobus solfataricus*. [1]

This categorisation seems to be very simple, but in fact there are a number of deviations from these two classes. It depends on method used for determination of the structure. There are many reports describing PNPs from both bacterial and mammalian sources with subunit compositions other than trimers or hexamers. It is usually difficult, for

example, to distinguish between pentamer and hexamer with experimental data of 125 kDa for $M_{r,}$ when a subunit mass is 24 or 25 kDa. [1]

PHYSICO-CHEMICAL PROPERTIES

pH

All PNPs, with very few exceptions, show broad pH activity optima between pH 7 and 8, or even 6 and 8.5, depending on the source of enzyme. The most striking exceptions are PNP I and PNP II from *Bacillus stearothermophilus* (pH neutral and pH 11) or *Brevibacterium acetylicum* (pH 8.5) On the other hand, acidic pH is optimum for xanthosine phosphorylase from *E. coli* or PNP from chicken liver (pH 6). Activity optima for E. coli and calf spleen PNPs correspond to the pH-dependent stability profiles of these enzymes. In general, high molecular mass PNPs are more thermostable than low molecular mass ones. [1]

Isoelectric point

Isoelectric points are in pH range from 4.2 (PNP from human mitochondria) to 6.8 (PNP from *Plasmodium lophurae*). Isoelectric point is important fact in kinetic behaviour of PNPs. Human PNPs of different tissues show electrophoretic heterogeneity resulting from post-translational modifications, but in contrast, calf spleen PNP shows no electrophoretic heterogeneity. [1]

Spectra

Absorption in the UV is typical for most proteins, with absorption maxima at 278 - 280 nm. Considerable difference occurs in extinction coefficients of various PNPs, since some of them do not contain tryptophan residues in amino acid sequence. E.g. human erythrocyte and calf spleen PNPs are $\varepsilon_{1cm}^{1\%} = 9.6$ and *E. coli* and *Cellulomonas* PNPs have extinction coefficients of $\varepsilon_{1cm}^{1\%} = 2.7$ and 5.9, respectively.

Fluorescence emission spectra of *E. coli* (contains only tyrosine) and calf spleen enzymes (contain both tyrosine and tryptophan) show their maxima at 304 and 340 nm, respectively. These spectra were obtained from interaction studies of enzyme with substrates or inhibitors in solutions. [1]

SPECIFICITY

Natural substrates

The natural substrates of low molecular mass PNPs, as has been mentioned, are 6-oxopurines (guanine, hypoxanthine and xanthine) and their ribosides and 2′-deoxyribosides, whereas the high molecular mass enzymes additionally accept 6-aminopurine (adenine) and its nucleosides. Ado or/and Ade can also react with low-mm enzymes in phosphorolysis or/and synthesis like competitive inhibitors. This was observed at PNPs from Sarcoma 180 cells, *Proteus vulgaris*, or *Cellulomonas*. [1] It appears that adenine may bind with some mammalian PNPs, but however, reaction velocities are so low, that it is unlikely that PNP plays significant role in adenine metabolism in mammalian cells. [2] Several enzymes exhibiting stricter specificity to 6-aminopurine nucleosides were found and they are referred to as adenine nucleoside phosphorylases or Ado phosphorylases.

PNP from *E. coli* cells cultivated in the presence of xanthosine is homohexamer with specificity and sequence of the low-mm enzymes, but it accepts xanthosine as a substrate. Xao, with pKa~5.7 occurs in physiological pH as a monoanion, resulting from dissociation of N(3)-H bond, and remains still 6-oxopurine. But its mode of binding probably differs from that of neutral nucleoside. [1]

Analogues

Early studies (70′s-80′s) described the specificity of low-mm mammalian PNPs and also high-mm enzymes from *E. coli* and *Salmonella typhimurium* versus a variety of nucleosides analogues with structural differences in the purine base or/and sugar moiety. Subsequently, comparison of the enzymes from E. coli and mammalian sources (calf and human) revealed the existence of nucleoside analogues that are selective substrates only for *E. coli* PNP. These include 1-methylinosine, 1-methylguanosine,

In a study with PNP isolated from vegetative cells and spores of Bacillus cereus, no differences in substrate specificity were seen. The analogue, 6-mercaptopurine ribonucleoside, was active as a substrate, whereas 2,6-diaminopurine ribonucleoside had a little or no activity.[2]

Unusual substrates

Some nucleoside analogues, such as m^7Guo, MESG, NR^+, 7-(β-D-ribofuranosyl)Gua, and others are substrates of PNPs from various sources, and undergo nonreversible phosphorolysis, or have a K_{eq} so small that the reaction is essentially irreversible. Others are resistant to phosphorolysis, but their bases may be substrates in the reverse, synthetic pathway, e.g. allopurinol, 8-azapurines.

Although Ado is not a substrate of low-mm PNPs, m^7Ado and 3-β-Ado are substrates with a V_{max} almost twice that for Ino with the calf spleen enzyme. N(7) methyl analogues of Ino and Guo are excellent substrates, as are other 7-alkylguanosines, such as, ethyl, propyl, butyl, benzyl.[1]

Carbohydrates

The sugar moiety of purine nucleosides have 1-carbon bounded to 9-nitrogen of the purine ring in the β configuration.[2] Both low-mm and high-mm PNPs are able to cleave the glycosidic bond of purine nucleosides, with the pentosyl moiety attached not only at position N(9), but also at N(3) or N(7). [1]

PNPs react with both ribonucleosides and deoxyribonucleosides, although in some cases with different kinetic parameters. In testing activity of a variety of nucleoside analogues modified in the sugar moieties, it was observed that arabinose-1-phosphate could not replace R-1-P as a substrate for fish muscle PNP and the carbocyclic analogue of inosin, where oxygen of the furanose ring is replaced by methylene group, did not react with or inhibit PNP from H. Ep No.2 cells. [2]

Reaction catalyzed

It is well established that PNPs can catalyze these reactions:

Reversible phosphorolysis:

purine riboside + phosphate ↔ purine + ribose-1-phosphate

Irreversible arsenolysis:

purine riboside + arsenate → purine + ribose + arsenate

Pentosyl transfer:

purine^{1} + purine^{2}nucleoside ↔ purine^{2} + purine^{1}nucleoside

With natural substrates and some nucleoside analogues, where phosphorolysis is reversible, the equilibrium is thermodynamically in favour of nucleoside synthesis. With the human erythrocyte enzyme, and those from calf spleen and *E. coli*, the equilibrium constant (K_{eq}) is ~ 50. But in vivo, phosphorolysis is the predominant reaction, due to coupling with other enzymes. Most of the unusual substrates of PNPs undergo phosphorolysis/synthesis irreversibly, or with K_{eq} so small that the reaction is irreversible. [1]

When inorganic phosphate is replaced by arsenate, reversible phosphorolysis catalyzed by PNP becomes irreversible arsenolysis. [1] Arsenolysis leads to formation of a highly unstable intermediate, ribose-1-arsenate, that hydrolyses immediately upon formation to yield ribose and arsenate. [2]

Direct transfer of the ribose or deoxyribose moieties without the intermediary formation of a phosphorylated pentose is probably an overall result of both phosphorolysis and synthesis, directed in part by the relative affinities for the enzyme of two purine bases. Stereochemical considerations make direct transfer reactions unlikely. Several studies indicate that the reaction mechanism is sequential rather than Ping-pong. Attempts to demonstrate and isolate ribosylated intermediate were unsuccessful. [1, 2]

KINETIC PROPERTIES

Kinetics

Kinetic studies have been performed in many different laboratories and K_m values even for the same enzyme markedly differ from one to another. For example, the K_m for inosine with human erythrocytic PNPase has been reported at values ranging from 0.05 mM to 1 mM. It may be due to several influences; method of assay, method of separation and purification of enzyme, phenomenon of "substrate activation at high

substrate concentration"[1], whereas at low concentration the substrate activation is not apparent. A possible explanation is that a substrate activation results from a cooperative effect among enzymic subunits, wherein it is assumed that the enzyme has two catalytic sites that interact with each other. [2]

The low and high-molecular mass phosphorylases share one important property: they show non-Michaelis kinetics with one or more substrates. Such data are consistent with substrate-induced cooperativity. It is interesting that the K_m values for orthophospate are relatively similar and fall in the range of 0,3 to 1,5 x 10^{-3} *M* [2]

Table 1Kinetic constants for phosphorolytic and synthetic pathways of the reaction catalyzed by PNP from various sources [1]

Kinetics constants determined by fitting classical Michaelis-Menten kinetic equations:

PNP	Variable substrate and its concentration range (mM)		K_m (μM)
E. coli	Pi	0.1 – 0.5	120
	Ino	0.01 – 0.5	32
	Ino	nd	47
	Guo	0.01 – 0.1	~20
	Ado	nd	12
	R-1-P	nd	40
	dR-1-P	nd	100
Human erythrocytes	Ino	nd	45
	Ino	0.01 – 0.5	~28
	Guo	nd	12
	Ado	nd	650
	Pi	0.08 – 0.25	66
	Pi	0.25 – 2	423
Calf spleen	Ino	0.01 – 0.5	13
	Guo	0.01 – 0.1	11
	Pi	nd	~860

Mechanism

The catalytic mechanism has been well studied; the cleavage reaction is believed to proceed through an S_N1 mechanism with oxocarbenium nucleoside intermediate, with ordered substrate binding, and with product release as the rate-limiting step.

Although the active site residues of trimeric and hexameric PNPs show considerable differences, the mechanisms are thought to be similar, with kinetic isotope effects consistent with an S_N1 mechanism. [3]

Escherichia coli PNP

E. coli PNP is the most studied enzyme from the group of high molecular mass PNPs. Typical representative is the product of *deoD* gene, referred to as *E.coli* PNP-I. It has been found that incubation of E. coli in the presence of xanthosine (Xao, but no other base or nucleoside) leads to the appearance of a second enzyme. This enzyme is a product of an induced *xapA* gene and cleaves more efficiently 6-oxopurine nucleosides like Xao, Guo and Ino, but not Ado. In the literature, it has been variously referred to as xanthosine phosphorylase, inosine-guanosine phosphorylase or *E. coli* PNP-II. Here the latter is used.

SUBSTRATE SPECIFICITY

The nucleobase specificity depends on the source, from which the enzyme is isolated. Whereas the human enzyme is specific for 6-oxopurine ribo- and deoxyribonucleosides, the *Escherichia coli* enzyme PNP-I accepts additional substrates including both 6-oxo- and 6-aminopurine ribo- and deoxyribonucleosides, and it even cleaves Adenosine (Ado) more effectively than Ino and Guo. On the other hand, *E. coli* PNP-II is unable to accept Ado, whereas Xao is an excellent substrate. The substrate specificity of this xanthosine-induced enzyme differs from that of *E. coli* PNP-I. PNP-II does not cleave or synthetise Ado or dAdo, but catalyzes the phosphorolysis of Xao, Ino and Guo. Thus, the specificity of this *xapA*-encoded PNP appears to be closer to that of mammalian PNP than to that of *E. coli deoD* encoded PNP. [3, 4, 5]

Substrate specificity in sugar moiety is similar in both *E. coli* PNPs. Both enzymes cleave ribonucleosides, 2′-deoxy-, 5′-deoxy-, and 2′,3′-dideoxyribonucleosides. However, a significant difference is that PNP-II fails to bind or cleave purine arabinosides, while PNP-I shows some activity for arabinose sugars.

STRUCTURE

PNP-I is a homohexamer in crystal form, but its subunit composition in solution has obviously not been established. PNP-II also appears to be a homohexamer, but with some evidence of co-existence of a trimeric form. PNP-II subunit arrangement is qualitatively different from the "trimer of dimers" arrangement of PNP-I; it is better described as "dimer of trimers". [4] The hexamer may be regarded as a flat cylinder approximately 60 Å thick and a diameter of 100 Å. In the centre of the hexamer there is a channel of approximately 20 Å diameter, filled with 24 water molecules. The distance between the active centres of dimers is about 19 Å. A comprehensive view of the hexamer indicates, that the overall structure is better described as a trimer of dimers. [5] The monomers alternate in an up/down fashion around the disc, with three of the active sites near the top and three near the bottom. The structures of the three crystallographically independent monomers vary slightly within the hexamer and from complex to complex. [3]

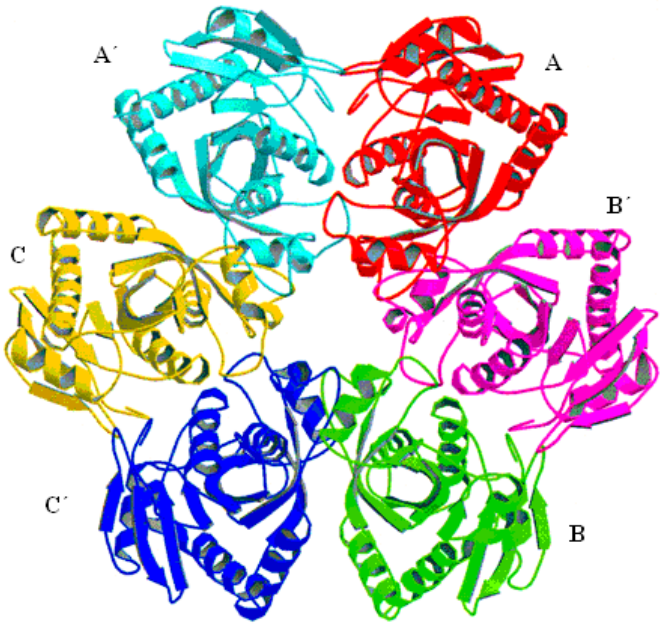


Fig. 5 Structure of the *E. coli* PNP hexamer. Three of the six monomers are crystallographically independent. The A, B and C monomers are generated from the A´, B´ and C´ monomers by crystallographic symmetry. [3]

CATALYTIC SITES

The core of each monomer consists of a nine-stranded mixed β-barrel, and is surrounded by eight α-helices, also found in mammalian PNPs, although there is no sequence homology between *E. coli* and mammalian enzymes. The active centre is located on the surface of each monomer and is exposed to solvent. It is composed of residues belonging to two neighbouring subunits [5] and consists of phosphate-binding site, ribose-binding site and purine-binding site.

Phosphate-binding site

The phosphate binding site is positively charged by three arginine side-chains and is the most buried part of the active centre. The phosphate binding site consists of Gly20 N, Arg24 N^{η}, Arg87 N^{η}, Ser90 N, Ser90 O^{γ} and Arg43 N^{η} from the adjacent monomer. All phosphate atoms form at least two hydrogen bonds or salt bridges with protein. The phosphate anion in monomers B or C has in principle similar contacts with the protein as in monomer A. Kinetic data show cooperative binding of phosphate to *E. coli* PNP, but since only one anion per monomer was found in the crystal structure, it supports the hypothesis, that there are two conformations of the enzyme with different affinities for phosphate. Because the phosphate binding site consists of residues belonging to two monomers forming a dimer, it is likely that binding of phosphate to one monomer affects conformation of the second monomer of the dimer, and thus the affinity to phosphate. [5]

Ribose-binding site

The ribose binding site is located very close to the position of phosphate anion, and all sugar oxygen atoms have direct contacts with it, except for O5′. The ribose binding site consists primarily of interactions with Glu^{181} and His^{4}. Ribose O5′ forms a hydrogen bond with His4 N^{ε} of the adjacent monomer and oxygens O3′ and O4′ are bridged by Glu181 O^{ε} and Ser90 O^{γ}, respectively. The latter is not observed in monomers B and C. [5] The 3′-hydroxyl group of 2′-deoxy ligands can interact with both carboxylate oxygen atoms, or maintain only the usual single (but stronger) hydrogen bond. Similar B-factors were observed for both compounds, and the K_m value for F-Ado was only

marginally lower than for F-dAdo. This suggests that loss of the O2´-hydroxyl group in ribose may not significantly affect substrate binding. [3]

Purine-binding site

The purine binding site consists of Ala^{156}, Phe^{159}, Val^{178}, Met^{180}, Ile^{206}, and Asp^{204} and it is largely exposed to solvent. The first four of these residues form a hydrophobic pocket around the purine base. Phe^{159} and Met^{180} are located between the purine base and the hydrophobic face of the sugar. Phe^{159} makes an angle of ~60° with the plane of the purine ring. [3]

A network of water molecules is observed, starting at the ring atom N(1) of the base and the exocyclic O(6). In addition, there is an ansembly of π-interactions between the base, Phe159 and Tyr160, which contribute to stabilization of peptide tertiary and quaternary structures. All interactions in the base binding site are unspecific, which explain the observed broad specificity of PNP of *E. coli* towards purines. For example, *E. coli* PNP is able to bind the purine riboside that has no hydrogen bonding substituents at the purine ring, as well as the riboside of benzimidazole, which lacks nitrogen atoms at positions N(1) and N(3). This supports the observation that aromatic-aromatic interactions with Phe159/Tyr160 direct the base into its binding position. [5]

For *E. coli* PNP, the leaving purine base is stabilized by a hydrogen bond from protonated Asp^{204} to purine N7. As the glycosidic bond begins to cleave, the resulting oxocarbenium ion is probably stabilized by the negatively charged phosphate group, since no amino acid side chain is available for this purpose. The reaction is completed by bond formation when the phosphate ion captures the oxocarbenium intermediate. [3]

ROLE IN CHEMOTHERAPY

The differences in substrate specificity between the hexameric and trimeric PNPs suggest a strategy for anticancer suicide gene therapy in which nontoxic nucleoside prodrugs are cleaved to cytotoxic purine analogues. [3] *In vitro* studies showed that melanoma cells transfected with the *E. coli* PNP gene convert non-toxic 6-methylpurine riboside into the highly cytotoxic purine base, which, as a membrane permeable agent,

mediates effective killing of neighbouring cells. [5] To optimize PNP anticancer gene therapy, the prodrug should be completely inert with respect to human enzymes and efficiently cleaved only by tumour cells expressing *E. coli* PNP. [3]

1.3. THYMIDINE PHOSPHORYLASE

Thymidine phosphorylase (TP) is an enzyme, catalyzing the phosphorolysis of 2´-deoxypyrimidine nucleosides. TP belongs to a family of *N*-ribosyl phosphorylases, but it is highly specific for the 2´-deoxyribonucleosides of thymine and related pyrimidine bases, whereas uridine phosphorylase (UP) doesn't distinguish between ribose and deoxyribose in pyrimidine nucleosides. [8, 7] TP catalyzes the reversible phosphorolysis of thymidine as follows:

thymidine + phosphate ↔ 2-deoxyribose-1-phosphate + thymine

Although the reaction is reversible, nucleic acid homoeostasis is dependent upon the catabolic reaction which drives the salvage pathway. The salvage pathway ensures that the pyrimidine-nucleotide pool is sufficiently large for efficient DNA repair and replication. [10]

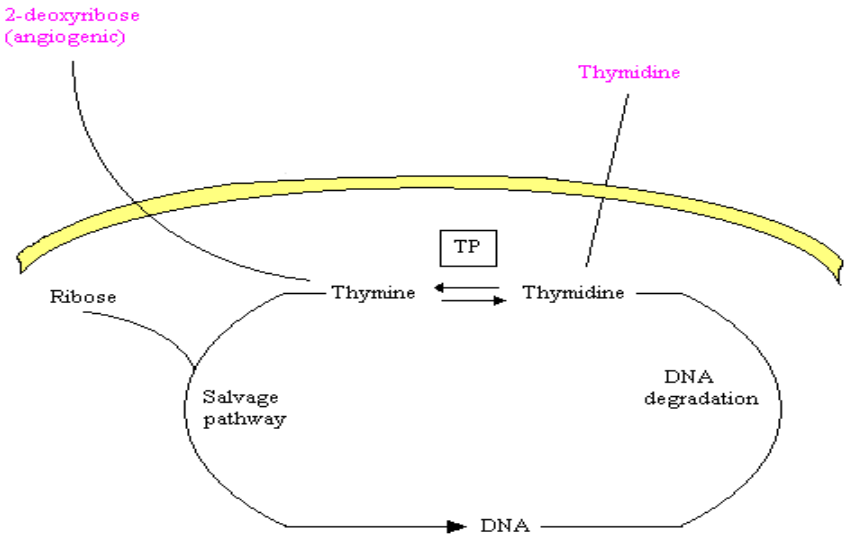


Fig. 6 TP catalyses the breakdown of thymidine to the base thymine and the sugar 2-deoxy-D-ribose-1-phosphate. The latter can only leave the cell once it has been dephosphorylated. TP clears cytostatic thymidine from cytoplasm and drives the nucleoside salvage pathway.

DISTRIBUTION IN NATURE

Thymidine phosphorylase is of widespread occurrence in microorganisms. [9]

In the 1970s TP was purified from both *Escherichia coli* and *Salmonella typhimurium*. In 1980s, a molecule extracted from human platelets was seen to have angiogenic effects and became known as platelet-derived endothelial-cell growth factor (PD-ECGF). Later, the angiogenic activity of 2-deoxy-D-ribose, a thymidine metabolite, was also reported. Subsequently, PD-ECGF and TP were confirmed as the same molecule. [6] The sequencing of PD-ECGF by Moghaddam and Bicknell revealed that PD-ECGF is the enzyme TP. [7]

THE ROLE IN HUMAN ORGANS, TISSUES AND METABOLISM

Angiogenesis is the term used to describe the formation of new blood vessels from the existing vasculature. In order to attract new vessels, a tissue must release an endothelial-cell chemoattractant. 2-deoxy-D-ribose is produced in vivo by the catalytic action of thymidine phosphorylase (TP) on thymidine (according to scheme above) and has recently been identified as an endothelial-cell chemoattractant and angiogenesis-

inducing factor. 2-deoxy-D-ribose appears to lack a cell-surface receptor, which is in contrast to majority of known endothelial-cell chemoattractants (mostly polypeptides) that bind to endothelial-cell-surface receptors.

TP expression is elevated in many solid tumours and in chronically inflamed tissues, both known areas of active angiogenesis. TP levels are higher in rheumatoid arthritis, in psoriatic lesions and lesser in osteoarthritis. High TP levels were found at the edge of gastric ulcers - a site of chronic inflammation which contains the highest concentration of inflammatory cells. A tumour is also a site of chronic inflammation. In some cancers, the invading macrophages can overexpress TP, but not the tumour cells themselves. TP levels in macrophages that have invaded colon tumours correlate well with microvessel density and are a good prognostic indicator.

There is evidence that TP is also involved in physiological angiogenesis such as endometrial angiogenesis during the menstrual cycle. The endometrium undergoes extensive angiogenesis during each menstrual cycle. TP is the only angiogenic factor whose levels have been shown to vary during the course of the menstrual cycle. The TP promoter contains half-palindromic estrogen response motifs, and endometrial TP expression is raised by the unique combination of progesterone and transforming growth factor-b1. [10]

TP expression is frequently raised in a wide range of human cancer tissues. [6] However, immunohistochemistry revealed that the cell type expressing TP varied between tumors. Thus, in some tumors, expression was restricted to the neoplastic element and in others it was restricted to the stromal or inflammatory component. [10]
Expression is tumour dependent, and depends on the presence of other nucleoside metabolism enzyme such as thymidylate synthase.

Thymidine phosphorylase also acts as an antiapoptosis factor. Because TP expression is very low in healthy human tissues, overexpression has been associated with the promotion of the tumour-genesis, whereas its genetic or pharmacological inhibition might suppress tumour progression. TP was shown to be associated with the anti-apoptosis mechanism, especially in cells damaged by stress, caused by factors such as hypoxia or chemotherapeutic drugs. [6]

ROLE IN CHEMOTHERAPY

Of the many nucleoside metabolism enzymes, TP is unique because it can both induce angiogenesis and protect cells from apoptosis. TP expression is stimulated by various types of anticancer treatments such as chemotherapy (taxanes, cyclophosphamide, oxaloplatine) or radiotherapy. Suppression of TP may slow down or suppress the recovery of cancer cells. Potential inhibitors of this enzyme could be useful in a treatment of some tumours, because they depend on the nucleotide salvage pathway for their proliferation. Also involvement in the phosphorolysis of different pyrimidine nucleoside analogues that can be used as antiviral or anticancer agents makes this enzyme interesting as a therapeutic target.

The best combination between TP-targeting therapies and TP-inducible treatments seem to have improved response and survival in patients with various types of cancer. [6]

STRUCTURE

TP is a dimer made up of two identical subunits with a dimeric molecular mass ranging from 90 kDa in *Escherichia coli* to 110 kDa in mammals. *E. coli* subunit is about 45 kDa and eukaryotic TP subunit normally has a molecular mass of 47 kDa. Human TP shares 39% of sequence identity with *E. coli*. [10]

BINDING SITES

Each subunit appears as a large α/β domain and a smaller α domain separated by a cleft. The phosphate ion is bound in the phosphate binding site in the α/β domain where it is stabilized by a salt bridge with Lys-84 and several hydrogen bonds with backbone amide nitrogens and hydroxyl groups from residues lining this pocket. The thymine base is bound in the thymidine binding site that is present in the α-domain, and its O4, N3, and O2 atoms are engaged in direct interactions with Arg-171, Ser-186, and Lys-190, respectively. There exist two forms of active site; open and closed, which are

separated by low-energy barrier. To generate a catalytically competent active site a domain closure is necessary. In this hinge-based closing motion there play roles Cα atoms of residues Leu-117 and Arg-171, which are placed in different domains.[8]

CATALYTIC MECHANISM

There are two studies, which deal with investigating of reaction mechanism of TP phosphorolysis using computer modelling. Each of them uses different program and method for observation and their results are in disagreement.

The study of Mendieta et al. [8] used molecular dynamics simulation and quantum mechanical calculation to find out the role of Histidine-85 in catalytic mechanism. They examined that His-85 can be involved in the transfer of the proton from the phosphate to the O2 thymine and also that the enol form of the free base is the most likely intermediate product, which then spontaneously tautomerize to a more stable keto form. They describe the whole process as two step mechanism. The first step is slower and rate limiting, during which the imidazol ring of protonated His-85 interacts with O2 in thymidine to make it a better leaving group. Weakening of the glycosidic bond generates an intermediate oxycarbocation in the sugar. The second step is a fast reaction between oxycarbocation and phosphate dianion, which acts as a nucleophile. These results support an S_N1 mechanism for the phosphorolysis reaction. [8]

On the other hand, the study of Birck and Schramm [7] also dealing with observations of transition state of TP enzyme using kinetic isotope effect analysis followed by computer modelling reveals, that human thymidine phosphorylase proceeds through an S_N2-like mechanism. The pKa of the 2-carbonyl oxygen of thymine is -2.98 which suggest that, at physiological pH, it is difficult to protonate this position to activate the leaving group. In TP, the activation of leaving group requires a higher degree of nucleophile participation for the reaction to proceed. [7]

1.4 USE OF ENZYMES AS BIOCATALYSTS

Purine nucleoside phosphorylase and also thymidine phosphorylase obtained from strains of *E. coli* may be used as a tool for enzymatic synthesis of nucleoside analogues with potential antiviral or antineoplastic activities. This applies particularly to those

instances where chemical synthetic procedures are tedious and inefficient. These procedures are useful for preparation of labelled nucleosides when the labelled purine is commercially available, but its desired nucleoside is not.

The general procedure for such syntheses involves the use of a pyrimidine nucleoside phosphorylase (uridine or thymidine phosphorylase) to release the α-D-pentose-1-phosphate from uridine or thymidine, which is then coupled by PNP to the desired purine base to give a purine nucleoside. Depending on enzyme specificity, the pentose ring may be ribose, 2′-deoxyribose, 2′,3′-dideoxyribose, arabinose or some other pentose. The entire reaction sequence is conducted in a single vessel, a so called "one-pot" synthesis. Although the kinetics of reverse synthetic reaction by PNP has been much less extensively investigated than the kinetics of phosphorolysis, the reverse pathway has been widely exploited for this purpose.

There exists studies, where PNP and TP enzymes from *E. coli* were employed for the synthesis of 6-dimethylamino-9-(β- D-deoxyribofuranosyl)-purine and 2-amino-6-chloro-9-β-D-ribofuranosylpurine each in 80% yield. A subsequent illustration of foregoing procedure is from the same laboratory. The α-D-2′,3′-dideoxyR-1-P, generated from 3′-deoxythymidine by TP from *E. coli*, was coupled to the desired 6-alkoxypurine by PNP (also *E. coli*).

TP and PNP were also employed in experiments of *trans*-5′-deoxyribosylation by PNP. The enzymes were used for the synthesis of the 5′-deoxy analogues of 6-thioguanosine and 6-merkaptopurine riboside, as well as the corresponding 2′,5′-dideoxy congeners, in yields of 40-50%.

An interesting variant of the general procedure, using only one phosphorylase, was based on the fact, that m^7Guo and m^7Ino are excellent substrates for PNP from *E. coli*. The products of the reaction, m^7Gua and m^7Hx do not markedly inhibit phosphorolysis, nor are they substrates in the reverse reaction. The reaction medium, therefore, contained m^7Guo (or m^7Ino) as the ribose phosphate donor, the desired purine base acceptor, and only PNP, which generated R-1-P from m^7Guo and coupled it to the desired purine. Three known nucleosides were synthesized in this manner in the yields of 60-100%. [1]

2. EXPERIMENTAL PART

2.1. AIMS OF THE STUDY

This work largely deals with two enzymes; Purine nucleoside phosphorylase (PNP) and Thymidine phosphorylase (TP). It mostly concentrates on initial evaluation of Biacore SPR analysis for enzyme-substrate interactions of the enzymes.

The first goal of the study was to test, if and how the enzymes produced by recombinant DNA technology can be bound on the carboxyl group containing CM 5 analytical chip of Biacore.

Secondly, it was studied, how and on which conditions the enzymes on chips bind with their natural substrates. The conditions varied were temperature, buffer, pH, molarity, and flow rate in the chip cell.

As natural substrates for PNP enzyme we used guanosine for the cleavage-directed and guanine with ribose-1-phosphate or deoxyribose-1-phosphate for the synthesis-directed reaction. For the TP enzyme, only the direction of phosphorolysis was studied, and in this its natural substrates thymidine, uridine and deoxyuridine were included. The whole reaction process took place in a Biacore X instrument.

The stability of an enzyme-coated chip was also evaluated by repeating the TP substrate binding assays after storing the chips refrigerated up to 10 weeks.

2.2. MATERIALS

2.2.1 Chemicals supplied by Biacore

Acetate buffer, pH 4.5 10mM Sodium acetate	Biacore AB, Sweden
Acetate buffer, pH 5.0 10mM Sodium acetate	Biacore AB, Sweden
EDC solution	Biacore AB, Sweden
Ethanolamine-HCl, pH 8.5 1.0 M solution	Biacore, Made in Scotland
Glycine-HCl, pH 2.5 10mM solution	Biacore, AB, Sweden
HBS-EP buffer, ready-to-use 0.01M HEPES, pH 7.4 0.15M NaCl 3mM EDTA 0.005% surfactant P 20	Biacore AB, Sweden
NHS solution	Biacore AB, Sweden
Mainatence Kit	Biacore AB, Sweden

2.2.2 Chemicals

2-deoxy-α-D-ribose-1-phosphate di(monocyclohexylammonium) salt	Sigma Chemical, USA
Acetic acid 99,8%	Riedel-de Haën, Germany
Deoxyuridine	Tintagel Limited, Hong Kong
D-ribose-1-phosphate Bis(cyclohexylamine) salt	(Fluka) Sigma-Aldrich, Canada
Guanine	Sigma Chemical, USA
Guanosine	Tintagel Limited, Hong Kong
PNP	from Prof. Miroshnikov, Inst. Mol. Biol., Sci. Acad. Russia, Moscow

Potassium dihydrogen phosphate	MERCK, Germany
Potassium dihydrogen phosphate	MERCK, Germany
Sodium acetate	MERCK, Germany
TP	from Prof. Miroshnikov, Inst. Mol. Biol., Sci. Acad. Russia, Moscow
Uridine	Tintagel Limited, Hong Kong
β-thymidine	Tintagel Limited, Hong Kong

2.2.3 Equipment

Biacore X, USA
pH meter ORION, model 420A, US

2.3. METHODS

2.3.1 THEORY

Immobilization procedure - amine coupling

The main steps for immobilization are generally the same. Immobilization of ligand to the sensor surface involves these three steps:

1. Activation of the sensor surface by appropriate reagents
2. Injection of ligand solution
3. Injection of reagent to deactivate remaining active groups and to remove non-covalently bound ligand. [11]

Amine coupling

This immobilization method is based on a reaction between amino and carboxyl groups. Dextran layer of the sensor chip provides carboxyl groups and the ligand must carry amino groups.

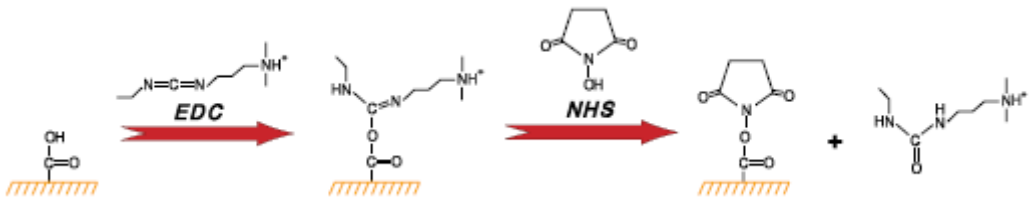


Fig. 6 Activation of the sensor surface by mixture of EDC/NHS – SensorSurface-Handbook (www.biacore.com)

At first, dextran surface is activated by mixture of *0,4M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)* and *0,1M N-hydroxysuccimide (NHS)* to give reactive succinimide esters. Then a ligand is passed over the surface and esters react spontaneously with primary amino groups or nucleophilic groups of ligand. The ligand is now covalently bound to the dextran matrix. The last step involves injection of *1M ethanolamine-HCl pH 8* to deactivate remaining active esters.

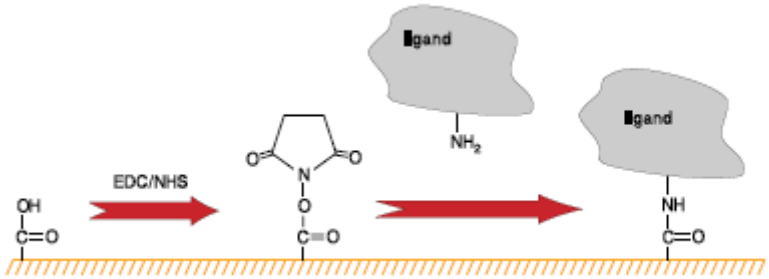


Fig. 7 Amine coupling of the ligand to the sensor surface - SensorSurface-Handbook (www.biacore.com)

Conditions for immobilization

Commonly used concentration of ligand is among 20-200 µg/ml. To get the high needed on the surface, a mechanism of electrostatic attraction of the ligand to the surface is necessary. Without this, the expected level of immobilization would be lower. This attraction is called as a *pre-concentration* and can increase the concentration of ligand on the sensor surface thousand-folds.

This pre-concentration is achieved by using appropriate pH environment. The carboxymethylated dextran matrix of the sensor chip carries a negative charged net at pH values above app. 3,5. The pH of the immobilization buffer should therefore be higher than this and lower than isoelectric point of the ligand.

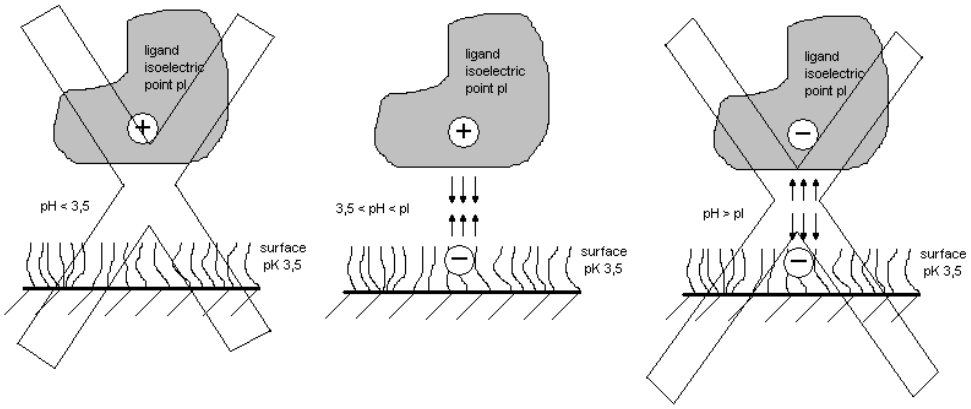


Fig. 8 Ilustration how pre-concentration influence the immobilization procedure – SensorSurface-Handbook (www.biacore.com)

Immobilization levels

The binding capacity of the surface depends on the level of immobilized ligand and it could be described in terms of the response at saturation as maximum response (R_{max}). A theoretical value of R_{max} can be calculated using the following formula, but usually experimentally derived R_{max} is lower for the same reaction. It may be caused by the fact that the ligand is not fully active, or there could be a sterical hindrance.

$$R_{max} = \frac{analyteMW}{ligandMW} * immobilized\ amount * stoichiometric\ ratio$$

But requirements of R_{max} differ from purposes of analysis. A low R_{max} is often beneficial in kinetic analyses, while higher levels are advantageous in concentration measurements.

Interaction analysis

Biacore X enables different types of assays. They can be divided into two main groups: Direct binding assays and Indirect assays. Direct assays involve the simple binding of analyte from the sample to the ligand immobilized onto the surface of the sensor chip, and this type was used in the present study. The indirect binding of analyte involves the use of an enhancement molecule and results in a sandwich approach.
The indirect assays can be further divided to Inhibition assay (solution competition) and Surface competition assay.

Interaction analysis is the process involving binding of analyte to the ligand and subsequent dissociation of analyte/ligand complex. Analyte is bound to the ligand during the injection and it is monitored in real time on the screen. After the analyte injection, when buffer flows over the surface, the dissociation of the analyte/ligand complex is monitored.

Fig. 9 Simple scheme of interaction analysis sensorgram – www.biacore.com

During the injection of a sample, when buffer environment greatly differs from the running buffer, a bulk effect on refractive index occurs, but it doesn't affect the binding. If the sample is diluted in the running buffer, this bulk shift is minimized. Role of the reference cell is central in correcting the result of an analysis for this bulk effect. The bulk contribution is subtracted from the analytical reflectance as illustrated in Fig. 10. The Biacore X Control Software can automatically subtract the bulk contribution.

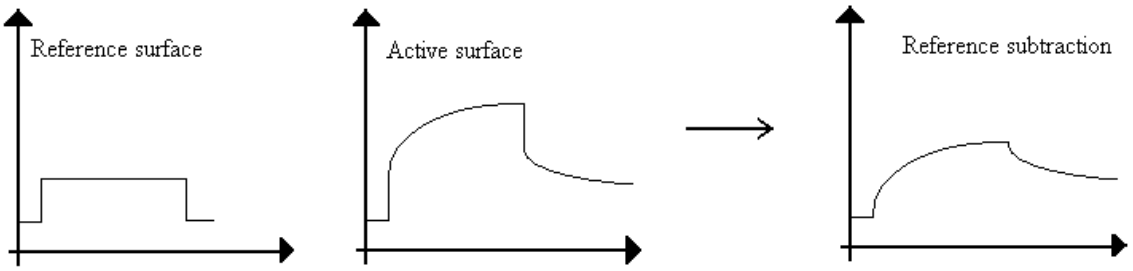


Fig. 10 The effect of reference subtraction – Biacore X; Getting Started (edition October 2002)

Although the reference-subtracted sensorgram shows the actual binding response, it can not correct non-specific binding to the dextran matrix of the reference surface, and the binding is not necessarily identical on both surfaces.

Regeneration

Regeneration is a process of removing bound analyte from the ligand after interaction analysis. This "washing" prepares surface of the sensor chip for the next analysis cycle. Process of regeneration can be repeated many times; it depends mainly on nature of the

attached ligand. One chip can be usually regenerated 100 times or even more than 1000 times.

The choice of conditions for regeneration is also dictated by nature and stability of ligand and analyte. Different applications may need individual regenerating conditions, but most surfaces can be washed with brief pulse of acidic (glycine-HCl) or basic (NaOH) solutions.

When the ligand is attached to the surface, regeneration usually doesn't destroy the ligand activity, but efficient reconditioning is very important for further successful assays. Incomplete regeneration or loss of binding activity will decrease repeatability and the lifetime of the sensor chip will be shortened.

If the sensor surface is not efficiently regenerated with an extreme pH, other conditions may be tested either alone or in combination with high or low pH. These include:

- up to 100% ethylene glycol
- high ionic strength (e.g.≤ 5M NaCl or ≤ 4M $MgCl_2$)
- low concentration of SDS (≤0,5%)

Sometimes it is not necessary to run regeneration. If the analyte dissociates fast enough, all analyte can be washed out with running buffer in a reasonable time. This is immediately evident from the sensorgram, since the response returns to the baseline after the sample injection.

Loading the sample

To avoid dispersion of the sample at the beginning and at the end of injection, a special technique of loading the sample is required. It is recommended to introduce small air and sample segments (about 5 µl each) into the pipette tip before loading. This separates the sample from running buffer and prevents mixing.

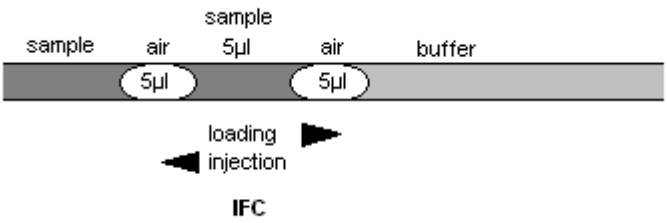


Fig. 11 Air bubble technique for loading the sample – Biacore X; Getting Started (edition October 2002)

Approximately 20 µl remains in the dead volume between the injection port and the loop. It doesn't come to the flow cells, so the minimum of the loading volume has to be raised by 20 µl. For clarity, the required total volume is shown at the bottom of the operation dialogue box of the instrument.

BIAevaluation Software

BiacoreX Control Software includes an interface to BIAevaluation to help in interpreting sensorgram data. BIAevaluation is used for calculations and creation of solvent correction plots. It also supplies kinetics and affinity measurements (calculating kinetics and affinity constants).

This software provides functions for:

- Comparing sensorgrams from separate runs by generating normalized overlay plots
- Deriving kinetic rate constant from association and dissociation phases of sensorgrams
- Deriving binding rates from sensorgram data, primarily for use in concentration measurements (from analyses under conditions where the binding rate is directly proportional to the analyte concentration)
- Evaluating concentration measurement

In this study, the program was used mainly for evaluation, and more close comparison of the sensorgrams from reference and active cell, because sometimes the response level was so low that it was difficult to see any binding.

Steps in the present BIAevaluation:

1. The current file was opened and only the curves from **Fc-1** and **Fc-2** were chosen (flow cell 1 is the reference cell and flow cell 2 is the active one).
2. The peaks of regeneration solution were removed by command **Edit: Cut**
3. Then the curves were aligned to the same starting point by **Calculate: X-transform → Curve Alignment**
4. At the last step the curves were adjusted to a common baseline by **Calculate: Y-transform → Zero at Average of Selection**

The sensorgrams are now at the same starting point and adjusted to a common baseline, and the differences in the response between these two curves are clearly seen.

2.3.2 PRACTICE OF THE EXPERIMENTATION

Immobilizing the ligand - coating the chip with PNP (TP)

1. Preparation of enzyme solution

I weighed app. 50 mg of PNP (TP) powder, which consists of about 50 % of pure PNP (TP) and the rest consist of the buffer salts that were present during lyophilization of the protein. The salts are first replaced by acetate buffer, by dissolving the material in 1 ml of the buffer. Then I made desalting on the small chromatography column Econo-Pac 10DG Column manual:

a) Pour out the excess of column preservative buffer from the column.
b) Fill it with the 20 ml of needed buffer (till the 30 ml mark), snap off the bottom tip and let the buffer drain out until it reaches the top frit.
c) Pour in your sample solution in volume of 3,0 ml. If there is a less then 3,0 ml, add buffer to get total sample volume.
d) Allow the sample to completely run into the column and discard this first 3,0 ml eluted.

e) Pour in 4,0 ml of buffer to elute higher molecular weight component(s) while collecting the 4,0 ml fraction from the column. [14]

From this pure concentrated sample I prepared solution of required concentration. I diluted the sample and then I filtered it through microfilter 0,2 μm. Now the sample is prepared for coating the chip.

Biacore X Control Software includes procedures (implemented as pre-defined macros) for ligand immobilization. Instructions are given on the screen. Each step of the procedure is well-defined in window, including the choice of coupling, standard volumes and injection times.

2. Amine coupling procedure

Surface activation

For the amine coupling, surface must be first activated by mixture of N-hydroxysuccimide (NHS) and N-ethyl-N´-(dimethylaminopropyl)carbodiimide (EDC). Aliquots of stock solutions of these, available from Amine coupling kit, are mixed together immediately before injection. I used 60 μl of each chemical. Injection takes 7 minutes when flow rate is 10 μl/min and injected volume of mixture is 70 μl. The sample loop has to be loaded with at least 90 μl and air bubble technique has to be used.

Injection of ligand solution

The same net volume (70 μl) of prepared and filtered enzyme solution is injected into the loop, after finishing activation of the surface.

Deactivation

Deactivation of remaining active esters in the matrix requires injection of the same net volume of 1 M ethanolamine hydrochloride pH 8,5, ready-to-use from Amine coupling kit.

After finishing this last step, the final operating dialogue box advises to condition the surface with a short pulse of regeneration solution before interaction analysis.

Schematic sensorgram for amine coupling:

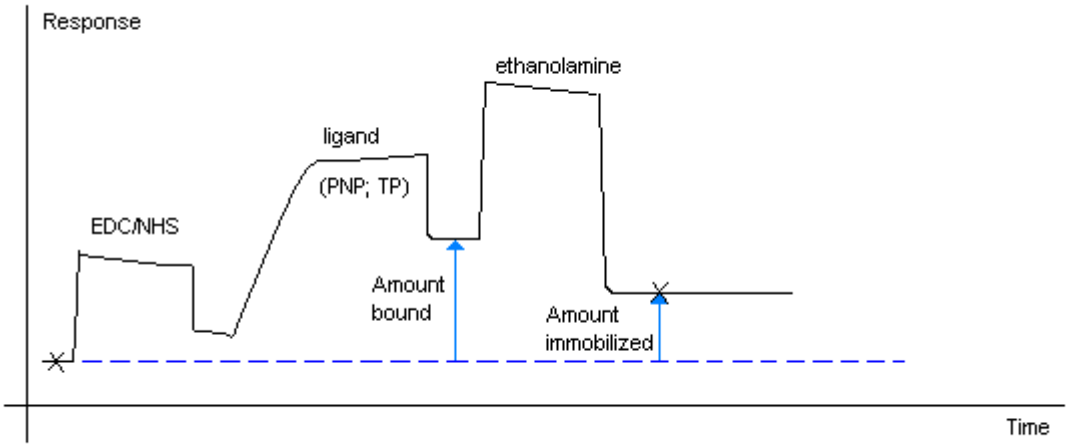


Fig. 12 A model sensorgram illustrating the distinction between the amount of ligand bound and the amount immobilized - SensorSurface_Handbook.pdf (www.biacore.com)

3. Interpretation of the sensorgram

When immobilization procedure has ended, the level of immobilized ligand is found out by using reference line and by adding report points at appropriate positions (Fig 11). At first the reference line must be dragged to the starting baseline of the sensorgram, where the first report point is added. This sets the baseline to zero for relative response at the other report points. Then the reference line is dragged to a position after ethanolamine injection and the second report point is placed there. This response level is given relative to the baseline response and represents the amount of the ligand covalently bound to the surface. Then the theoretical maximum binding capacity (R_{max}) can be calculated by using the equation described in theoretical part.

Interaction analysis

I was testing guanosine as a substrate for PNP in the direction of cleavage reaction and guanine and ribose-1-phosphate and deoxyribose-1-phosphate in the direction of synthesis. For the TP enzyme I tested mostly thymidine as a substrate, but I also tried uridine and deoxy-uridine in one case. I always prepared analyte solutions of two different concentrations, 1 mM and 0,1 mM, except in one experiment with thymidine,

where I used solution of concentration of 0.2 mM. Appropriate amount of the testing substance was weighed out and dissolved in 10 ml of buffer. Buffer was one of the variable conditions (see Variation of conditions). The lower concentration was achieved by diluting the 1 mM solution ten folds (1:10). These solutions were then filtrated through microfilter (0,2 μm) before injecting in the instrument.

Just as in immobilization, each step in interaction analysis is guided window by window through the computer-aided operating system. Instructions are given for choosing the flow path, flow rate and injected volume of analyte and regeneration solution.

Starting to run sensorgram begins with choice of parameters.

1. Detection mode - multichannel in order of flow cells 1-2. (Flow cell 1 is the reference cell and flow cell 2 is that one with immobilized PNP.)
2. Flow rate – 10 μl/ml

Then the injection of analyte solution follows in net volume of 30 μl. After finishing the interaction analysis, short pulse of regeneration solution (glycine-HCl, pH 2,5) is injected in net volume of 5 μl. Stopping of the sensorgram is possible when the response after regeneration is back on the baseline.

Variation of conditions

Buffers

The buffer plays two roles in experiments. It serves as a running medium through whole system and also as a solvent of analyte (Guo, Gua, etc.) Mostly the same buffer was used as running medium and as solvent, but in some cases different buffers were used for the two purposes.

Type of buffer used	*Molar concentration*	*pH*
HBS-EP buffer (available from Biacore)	10 mM	7,4
Phosphate buffer ($K_2HPO_4 + KH_2PO_4$)	varies from 1 mM to 50 mM	varies from 6,2 to 8,0
Acetate buffer (from Biacore or prepared)	10 mM	5,0

Specifications of buffers from Biacore are described in more details in MATERIALS.

Particular types of Phosphate buffer ($K_2HPO_4 + KH_2PO_4$):

	Molar concentration	**pH**
for PNP trials	50 mM	7,2
	10 mM	6,2
		6,7
		7,1-7,3
		8,0
	1 mM	7,1
for TP trials	50 mM	7,0
	20 mM	7,2
	10 mM	7,1
	2 mM	7,1
	1 mM	7,2
	0,2 mM	7,1

Temperature

Usually the basic laboratory temperature of 25ºC was used. In some trials, the temperature was increased to 30ºC and 35ºC to observe, whether it influences the activity of enzyme.

Flow rate

I used basic flow rate (10 µl/min) in most of the trials. While working with TP enzyme I tried to change the flow rate and see, if this has an influence on behaviour of the enzyme. One of the new rates was half speed (5 µl/min), and the second was double speed (20 µl/min).

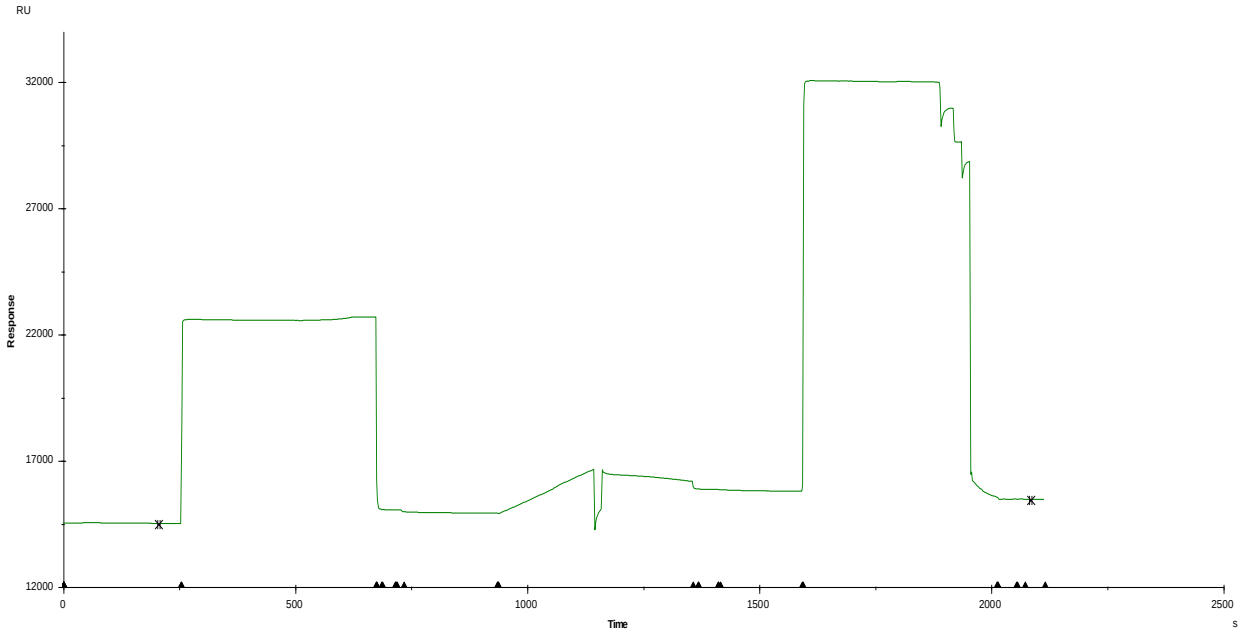
Summary of used conditions for each enzyme is in the following tables.

3. RESULTS

3.1 Results from experiments with PNP enzyme

3.1.1 Comparison of immobilizing procedure of PNP enzyme in three different concentrations to the surface of the sensor chip.

Sensorgram of immobilization of PNP (100 µg/ml) – 1st chip

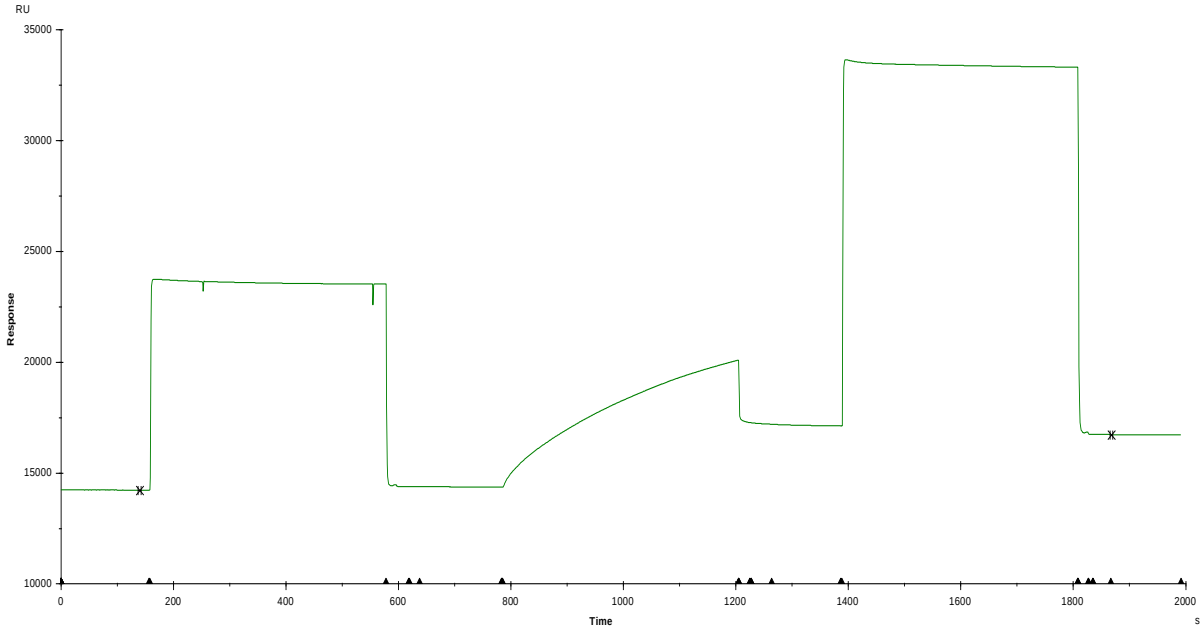


Report point table:

Cycle	Fc	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
1	2	205.5	5.0	14507.2	0.31	-0.14	0.19	Yes	0	baseline
1	2	2084.5	5.0	15458.0	0.43	-0.15	0.36	No	950.8	ethanolamine

This is the sensorgam and the report point table of immobilization procedure. The sensor chip was coated with solution of PNP in concentration 100 µg/ml dissolved in 10 mM acetate buffer, pH 4.5. The first peak represents activation by EDC/NHS mixture, the second peak represents immobilization of PNP and the third one is deactivation of remaining active esters. The drop during immobilizing procedure is caused by air bubble, which changed medium in detecting unit and subsequently the response in reflection. The sensorgram indicates that the immobilized level is quite low, only 950.8 RU.

Sensorgram of immobilization of PNP (300 µg/ml) – 2nd chip

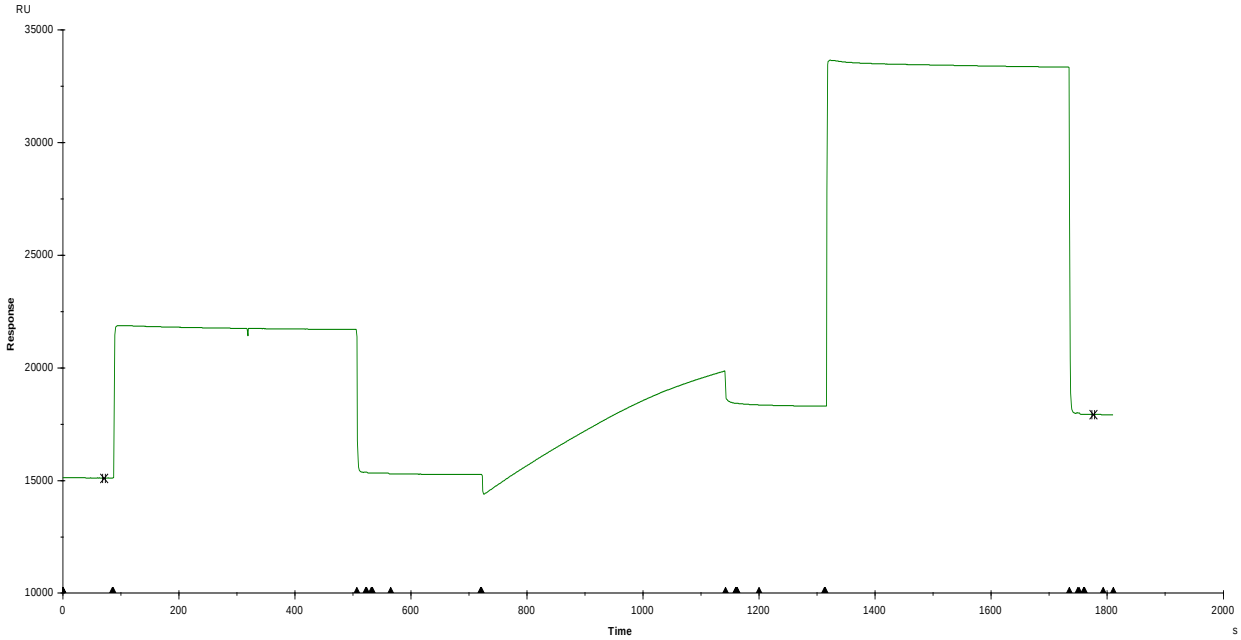


Report point table:

Cycle	Fc	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
1	2	140.5	5.0	14223.8	0.15	-0.06	0.12	Yes	0	baseline
1	2	1868.5	5.0	16725.5	0.38	-0.13	0.32	No	2501.7	ethanolamine

This is the sensorgram and the report point table of immobilization to the second chip. There I used higher concentration of PNP – 300 µg/ml on the same conditions – and also the level of immobilized enzyme was higher. In comparison with the first sample of enzyme, where the concentration was 100 µg/ml and immobilized level was 950.8 RU, the immobilized level here is 2.6 times higher.

Sensorgram of immobilization of PNP enzyme (600 µg/ml) – 3rd chip



Report point table:

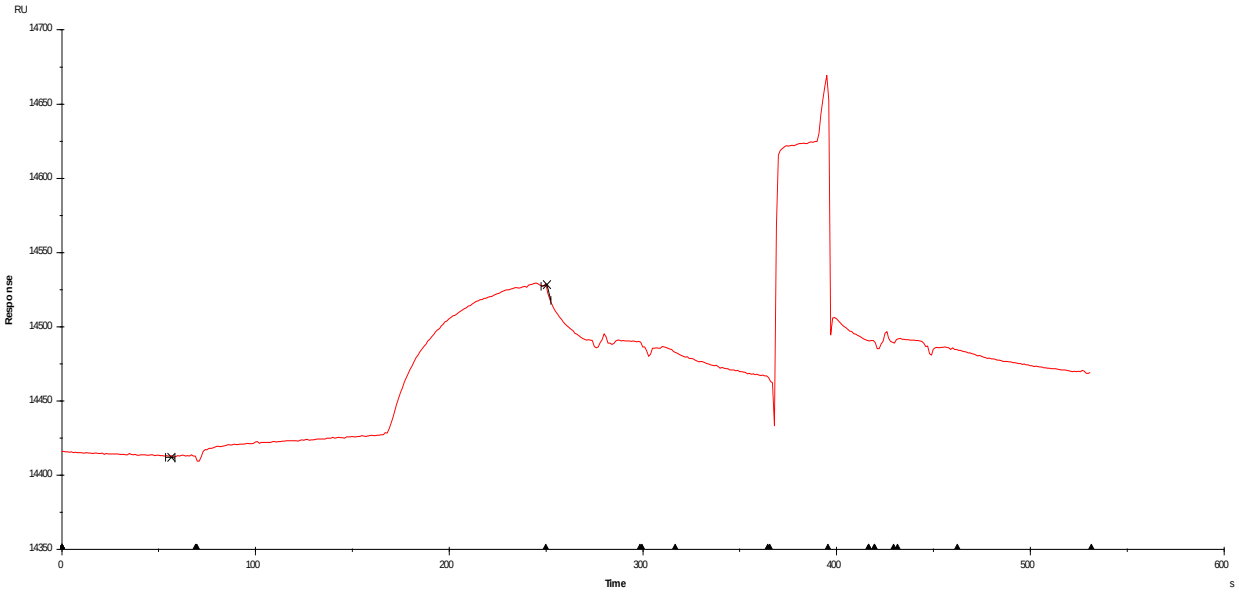
Cycle	Fc	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
1	2	71.5	5.0	15106.3	0.16	0.00	0.18	Yes	0	baseline
1	2	1776.5	5.0	17919.9	2.08	-1.02	0.92	No	2813.6	ethanolamine

This is the immobilization of third chip, where the highest concentration of PNP (600 µg/ml on the same conditions) was used. In comparison to the previous procedure, double increasing of enzyme concentration did not increase the immobilized level in the same rate. Relative response of the concentration 300 µg/ml was 2501.7 RU and for 600 µg/ml was it 2813.6 RU, which is only about app. 300 RU more.

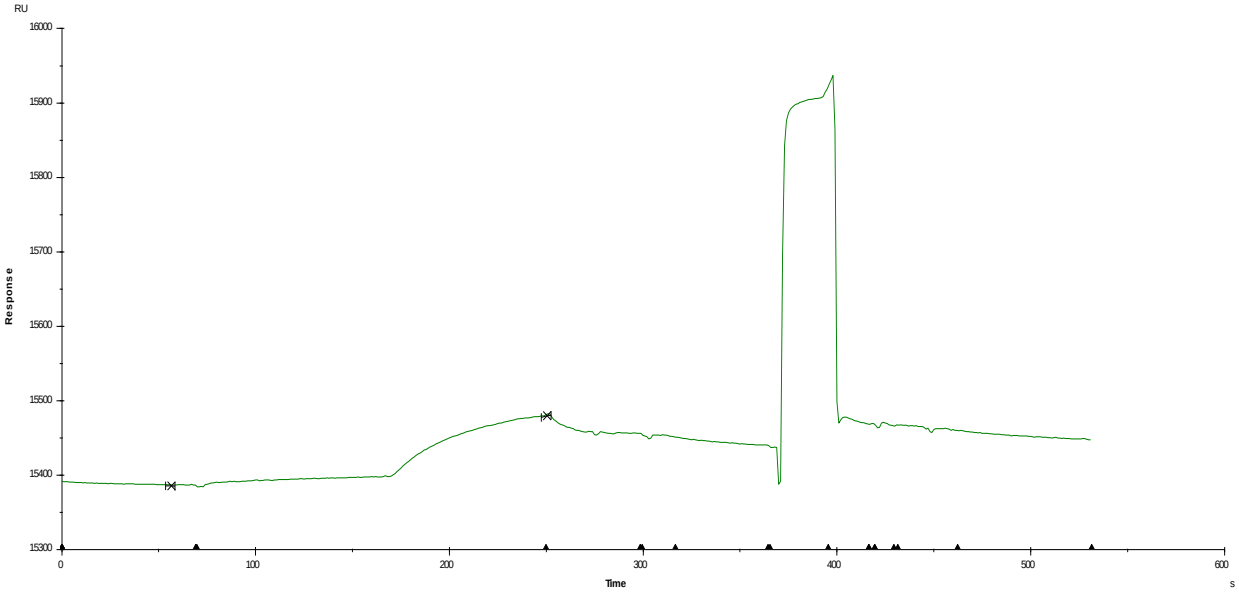
3.1.2 Interaction analysis between guanosine dissolved in phosphate buffer and PNP enzyme (100 µg/ml) immobilized on the surface of the sensor chip.

Sensorgrams of guanosine 0.1 mM in phosphate buffer 10 mM, pH 7.2

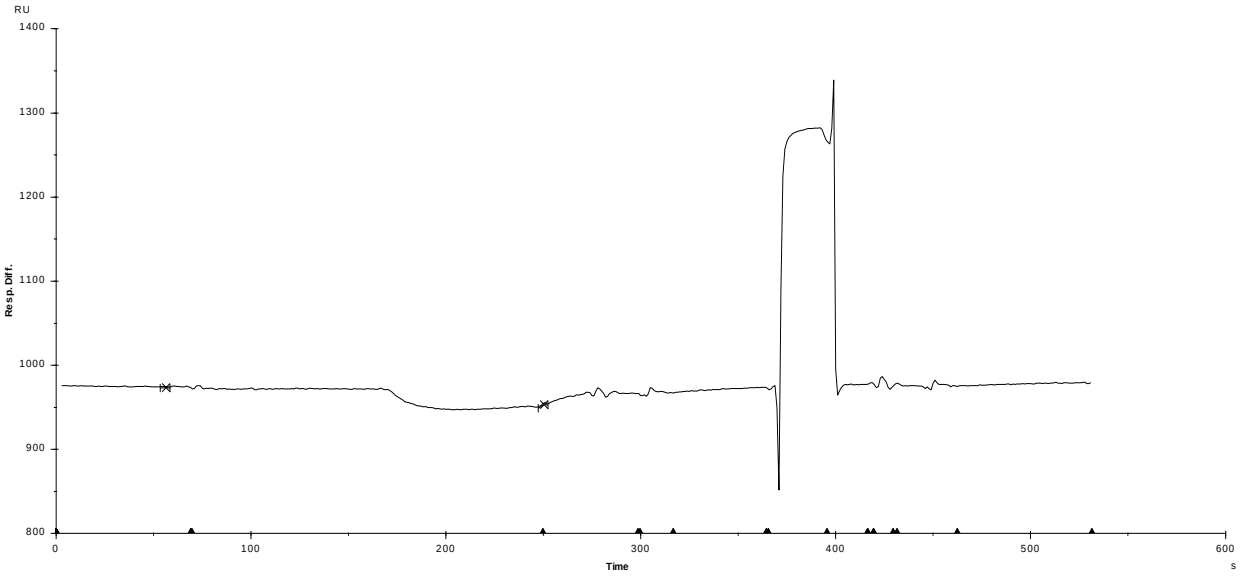
Fc 1 - reference cell



Fc 2 - active cell



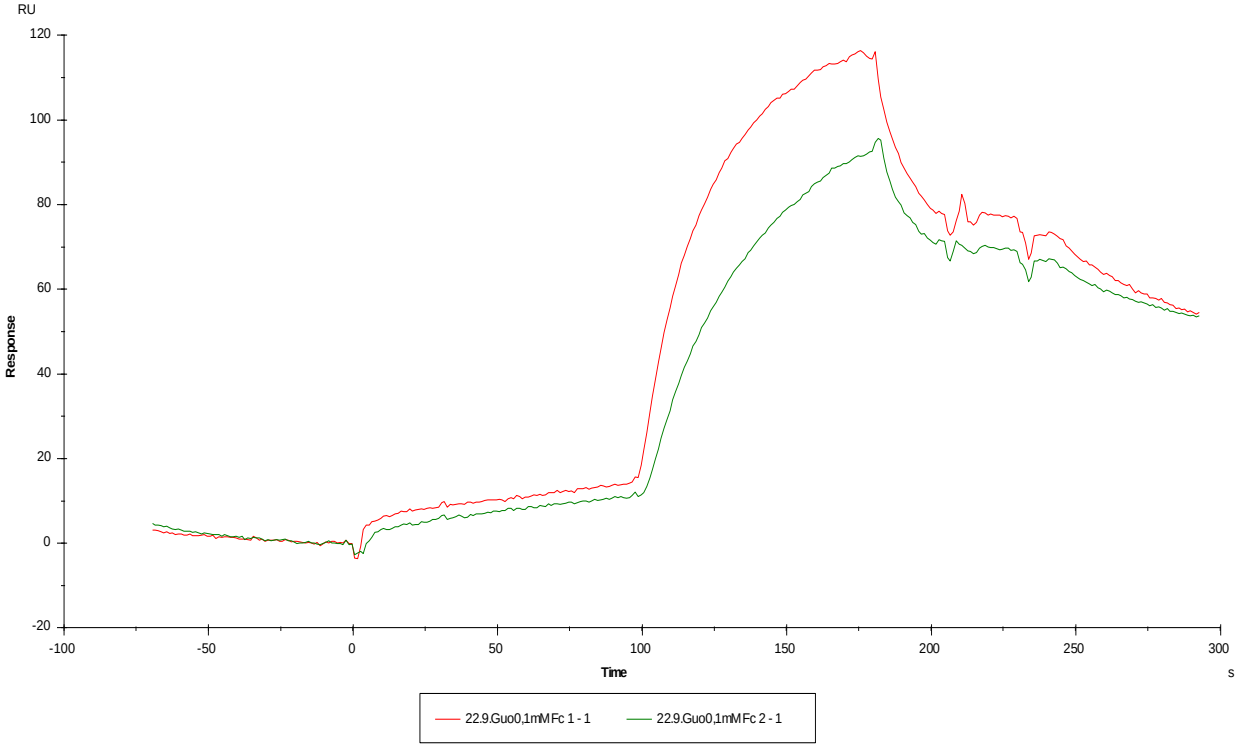
Fc 2-1 - subtracted curve



Report point table:

Cycle	Fc	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
1	1	56.5	5	14412.2	0.29	-0.1	0.25	Yes	0	base
1	2	56.5	5	15385.6	0.3	-0.13	0.18	Yes	0	base
1	2.1	56.5	5	973.3	0.21	-0.06	0.2	Yes	0	base
1	1	250.5	5	14524.8	4.12	-1.74	2.82	No	112.6	guo 0.1mM
1	2	250.5	5	15479.3	1.6	0.8	0.63	No	93.7	guo 0.1mM
1	2.1	250.5	5	951.7	2.17	1.02	1.17	No	-21.6	guo 0.1mM

BiaEvaluation plot of guanosine 0.1 mM in phosphate buffer 10mM, pH 7.2

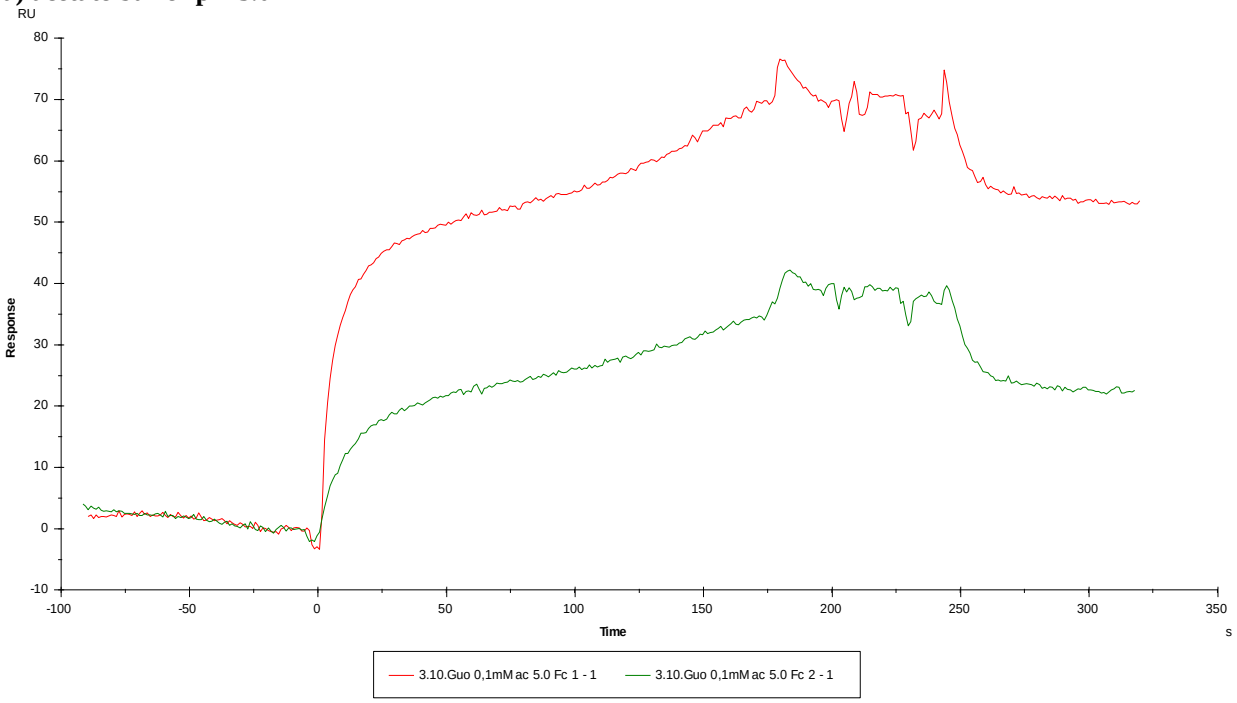


Curves show binding of guanosine to PNP in flow cell 2 and non-specific binding of guanosine to the dextran matrix on the surface of the sensor chip in flow cell 1, which serves as a reference cell. This confrontation shows that relative response from flow cell 1 is higher than that one from flow cell 2, which indicates that enzyme is either not active or the amount immobilized to the chip is insufficient, and only the binding of guanosine to the dextran matrix is observable.

3.1.3 Interaction analysis between guanosine dissolved in phosphate buffer of different pH values and PNP enzyme.

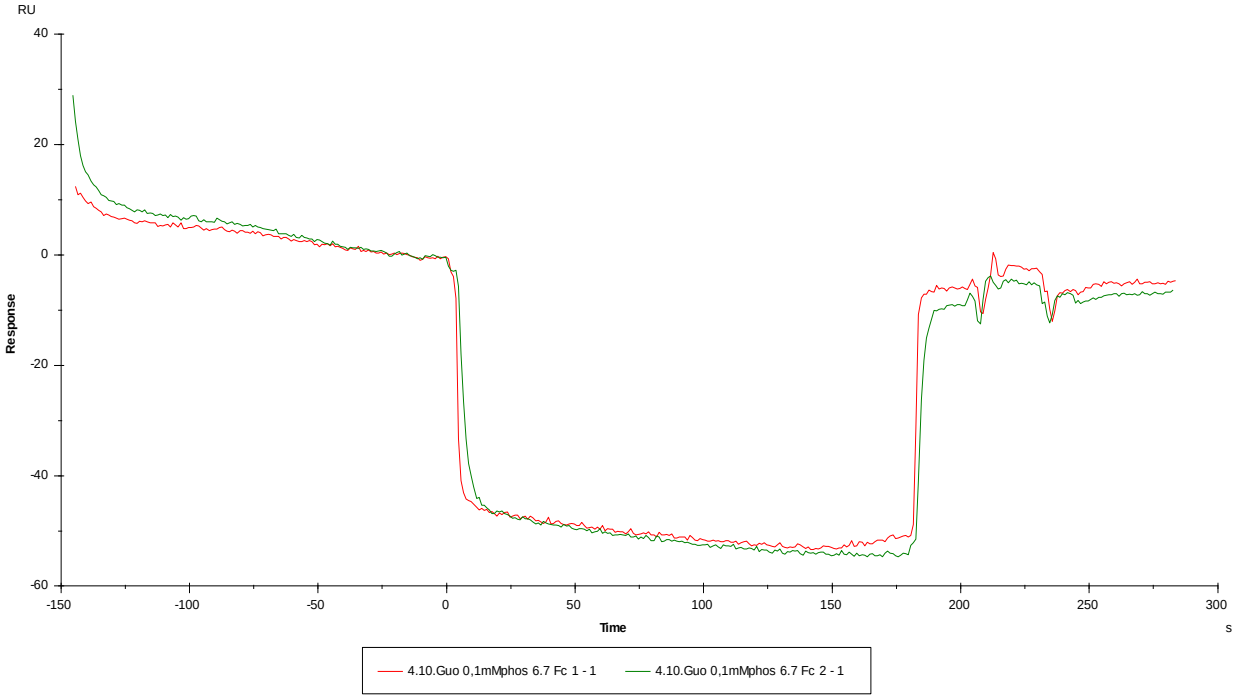
BIAevaluation plots of 0.1mM guanosine in 10mM buffers of different pH

a) acetate buffer pH 5.0



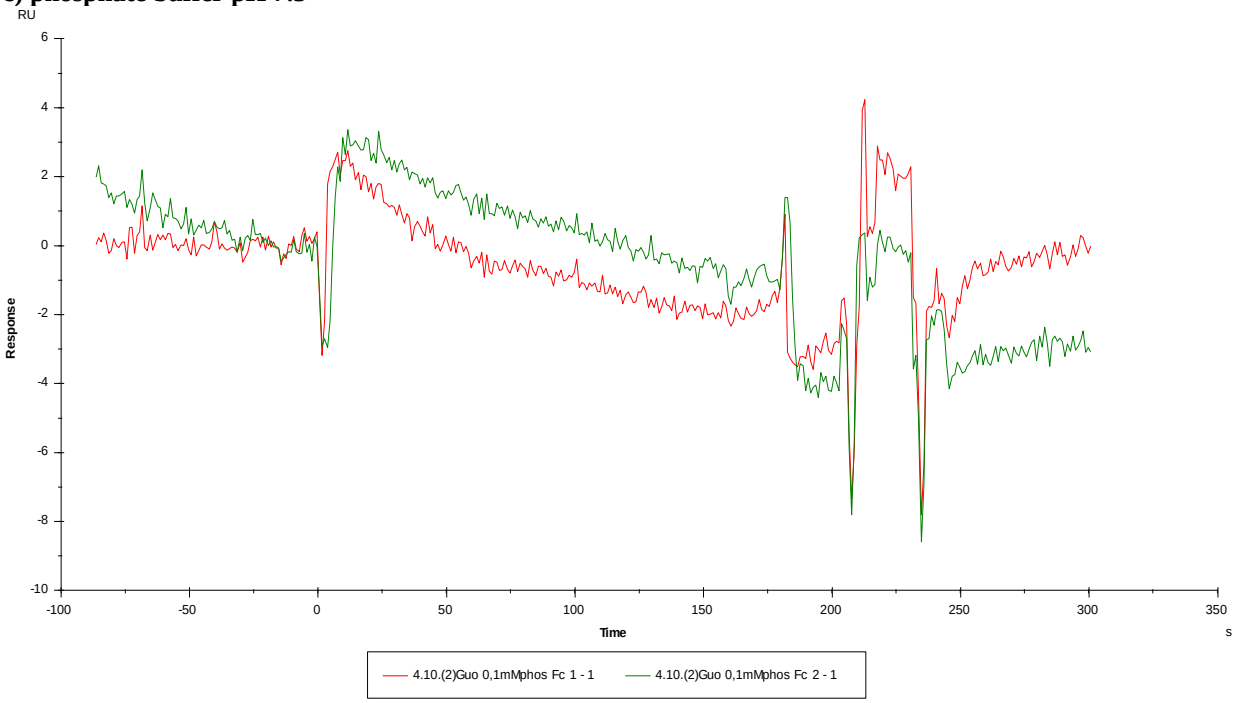
The shape of the curve shows binding somehow, but there is again higher non-specific binding in reference cell than binding to the active surface.

b) phosphate buffer pH 6.7



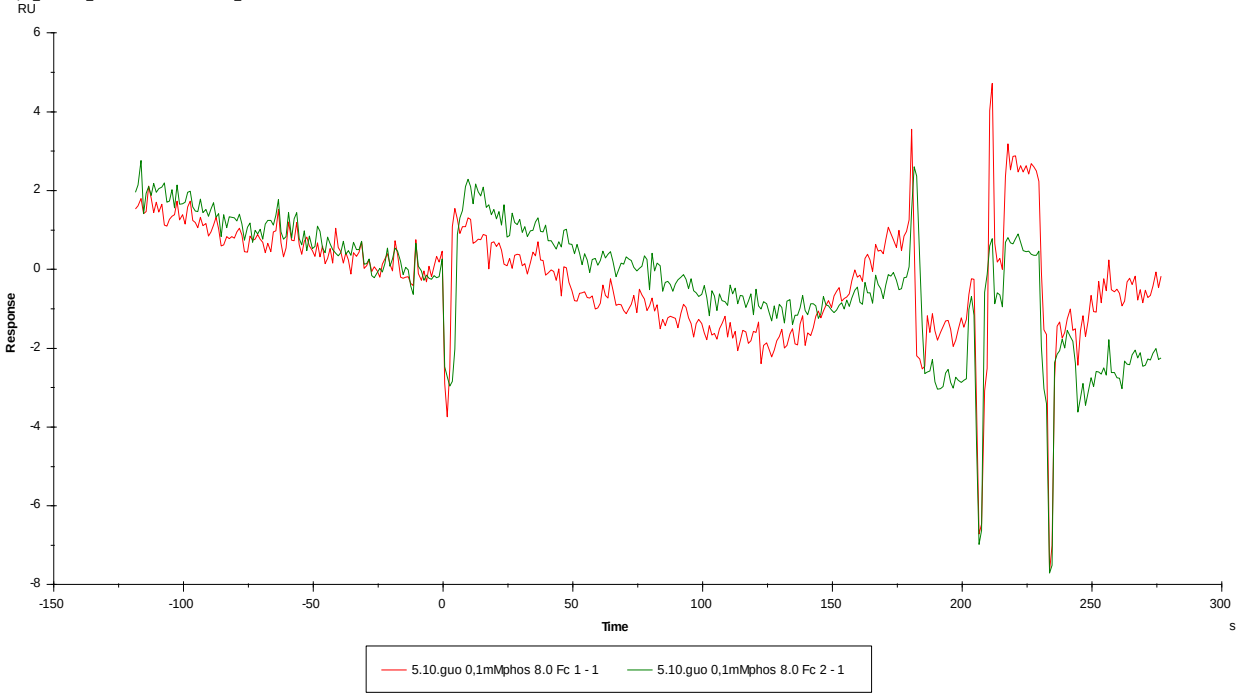
At this pH 6.7, a big drop occurred and the relative response was negative, -52.7 RU. Curves from both the reference and active cells show almost no difference in binding levels.

c) phosphate buffer pH 7.3



The binding at pH 7.3 and at pH 8.0 on these two plots looks very similar. The relative responses wave along the baseline for both the relative and active curves.

d) phosphate buffer pH 8.0

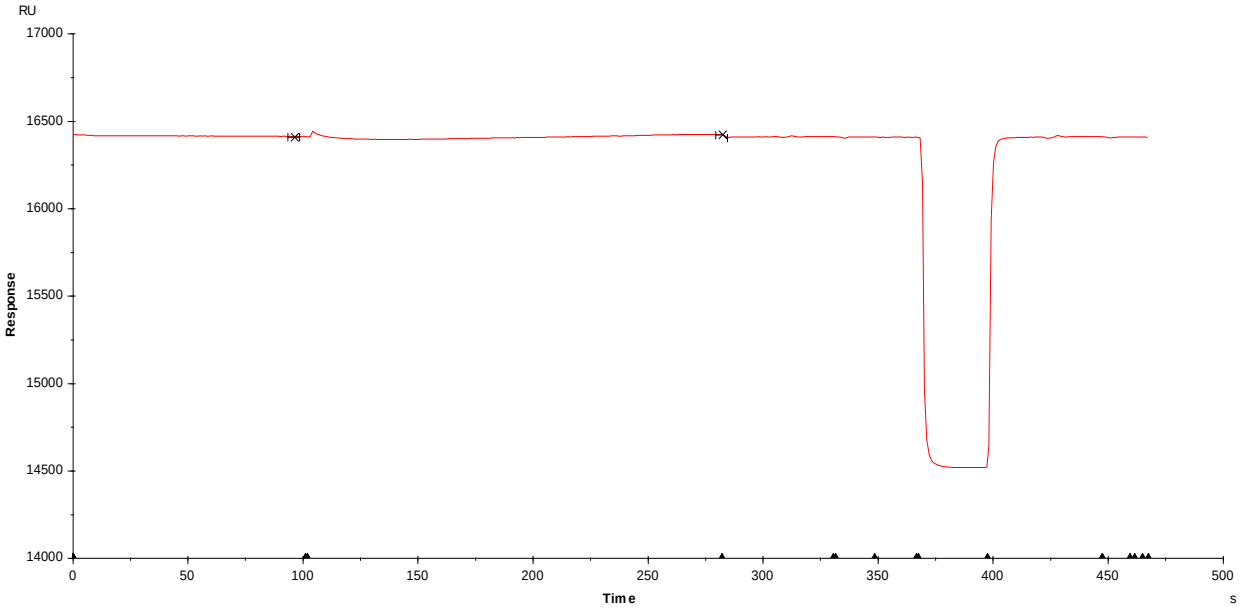


If we compare the BIAevaluation plots, it is hard to say whether and how the pH influences the binding of guanosine to PNP enzyme, because results vary a lot and there is no relation. The first two sensorgrams are absolutely different from others and the last two seem to be almost of the same shape – like there is minimal binding.

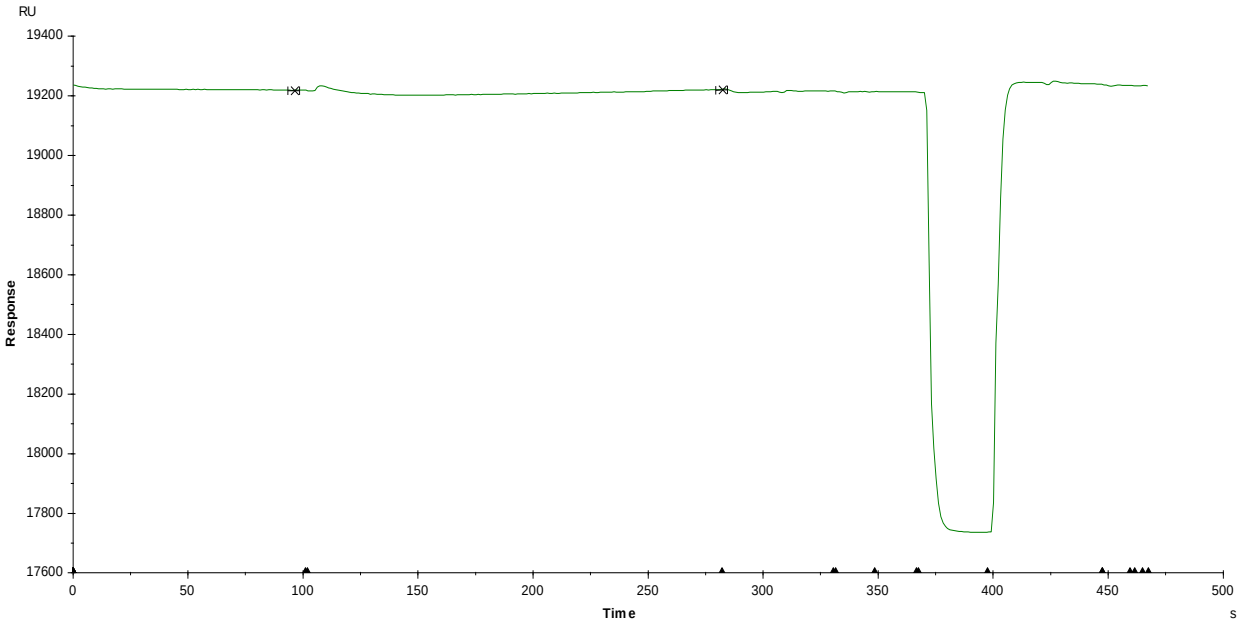
3.1.4 Interaction analysis between guanine "dissolved" in HBS-EP buffer with and without addition of DMSO and PNP enzyme.

Sensorgrams of 0.1 mM guanine in HBS-EP buffer

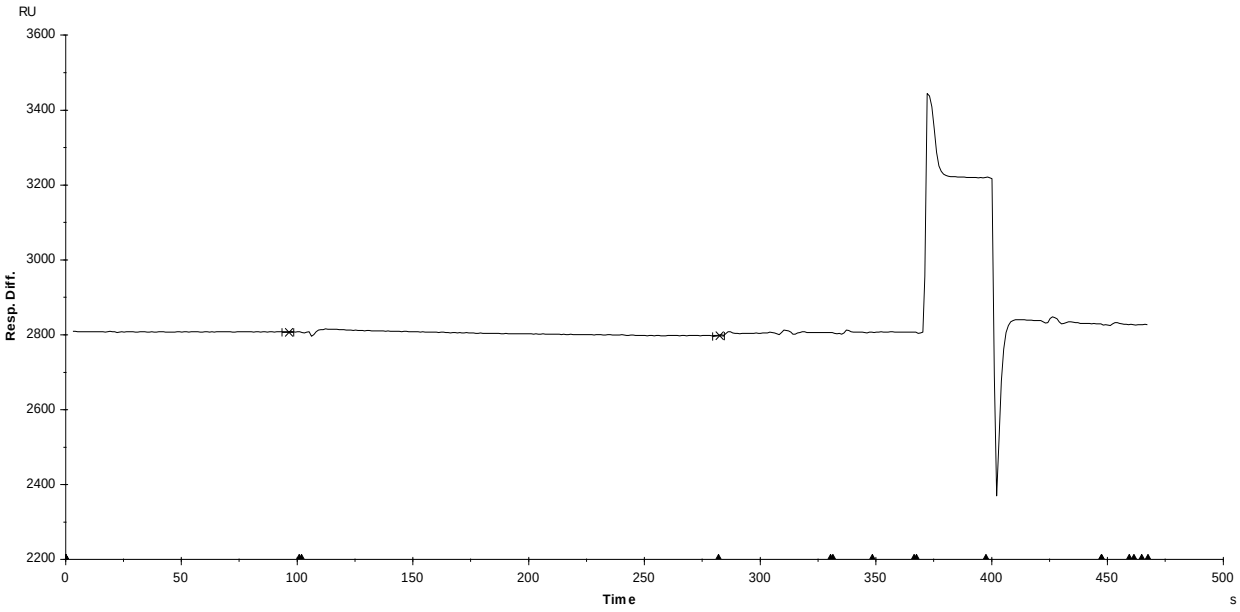
Fc 1 – reference cell



Fc 2 – active cell

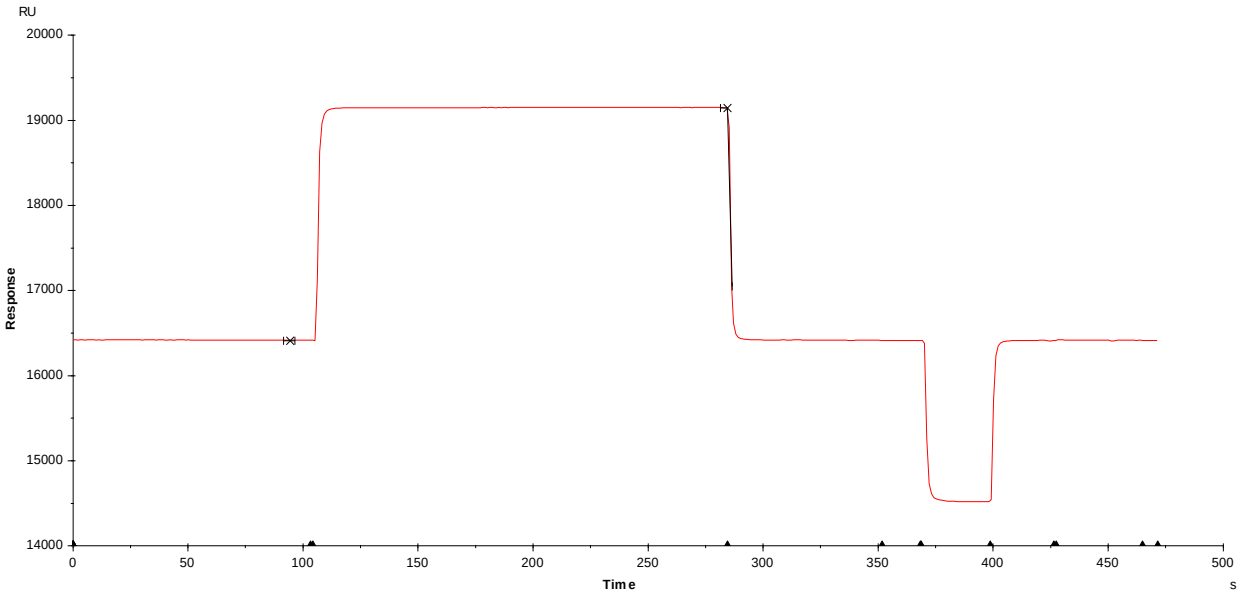


Fc 1-2 – subtracted curve

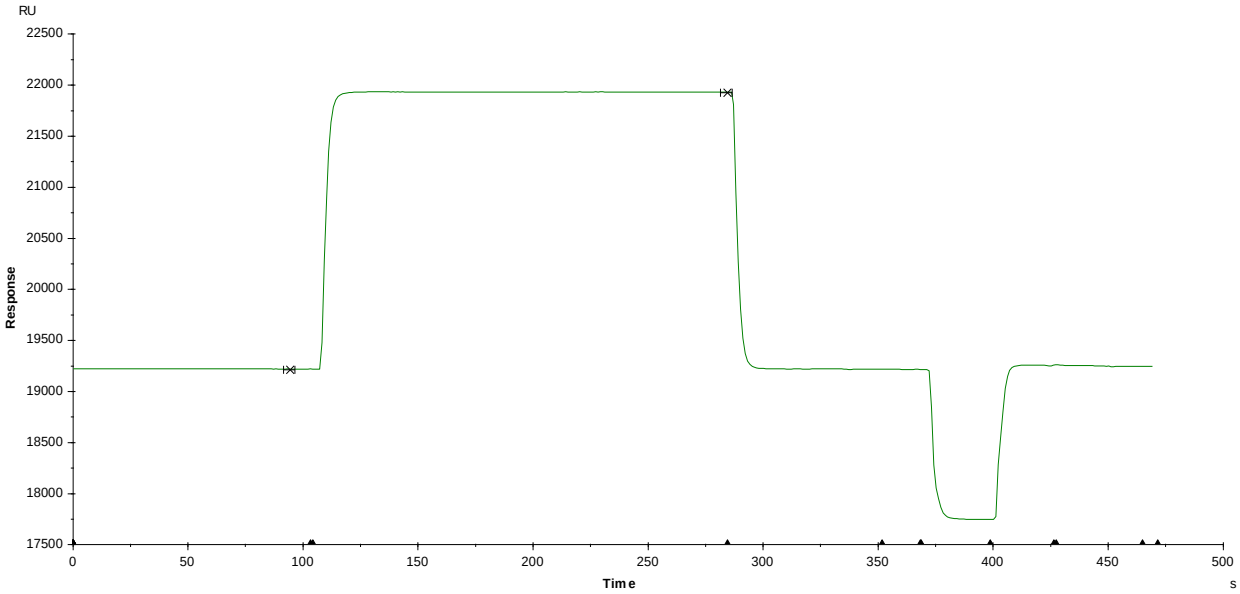


Sensorgrams of 0.1 mM guanine in HBS-EP buffer with 2% (V/V) of DMSO

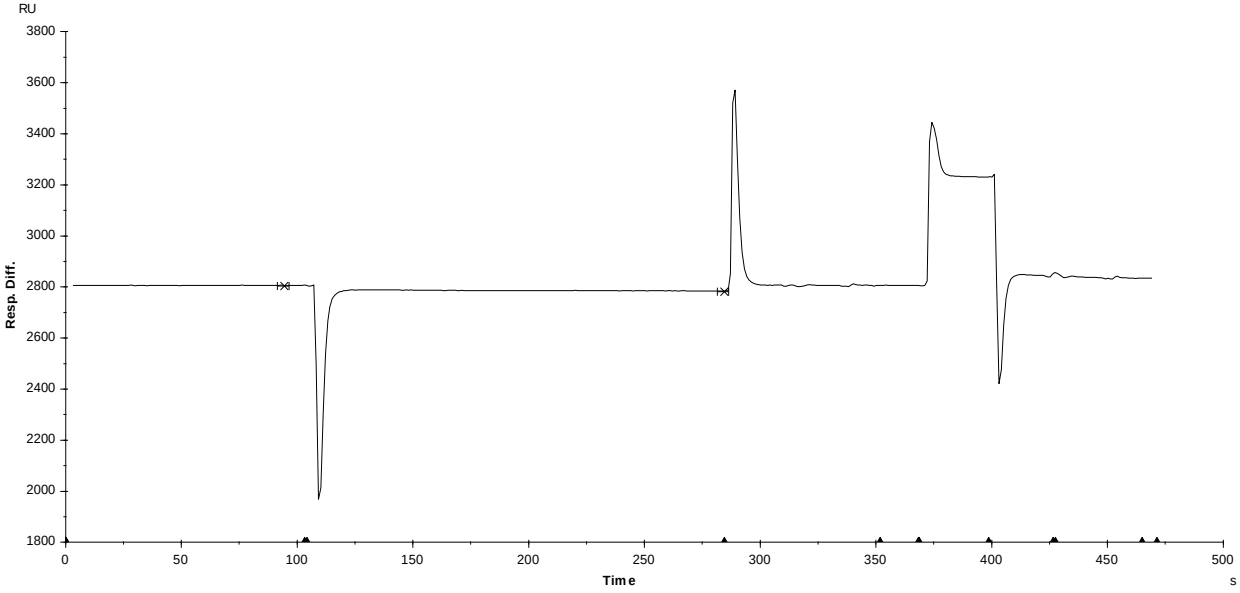
Fc 1 – reference cell



Fc 2 – active cell



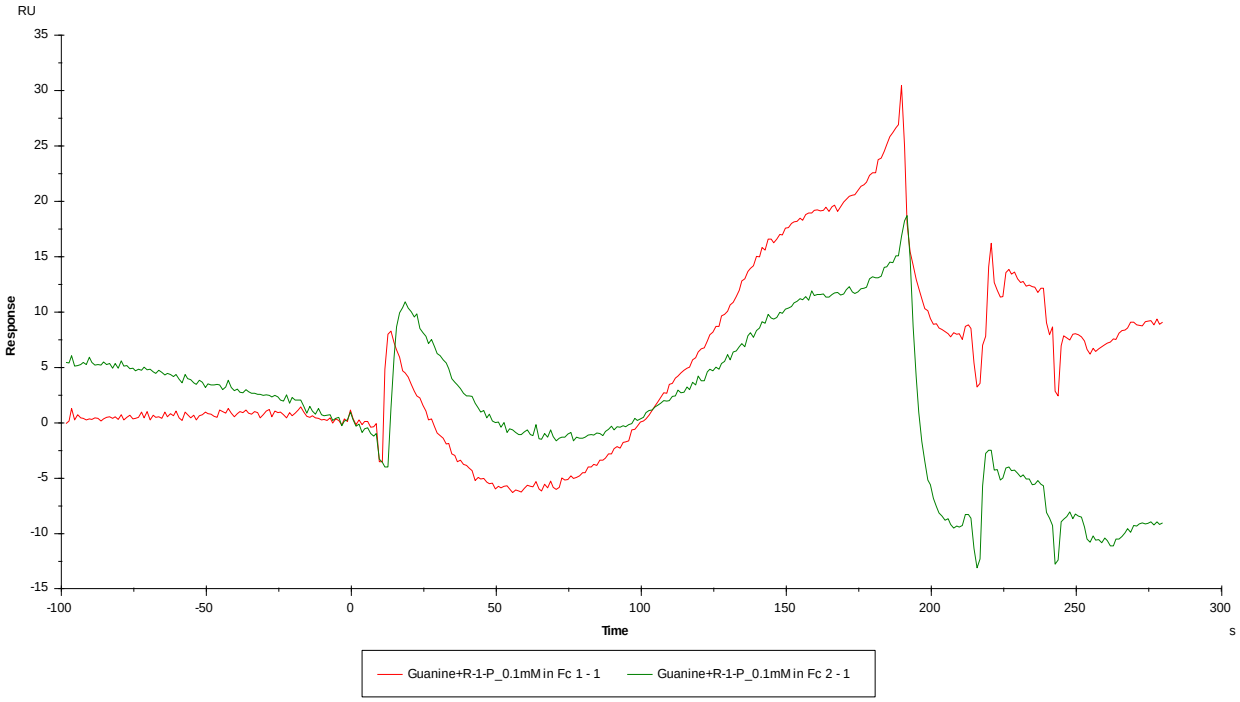
Fc 1-2 – subtracted curve



As sensorgrams clearly show, DMSO caused big change in the response and these great peaks in both of the cells totally covered /overlaid any binding of guanine. But the guanine was not dissolved even thought there was added 200 µl of DMSO to 10 ml of 0.1 mM guanine. So it is not known how much of the guanine was dissolved.

3.1.5 Comparison of BIAevaluation plots from interaction analyses between guanine and ribose-1-phosphate or deoxyribose-1-phosphate in one solution (two different concentrations) and PNP enzyme (300 µg/ml) fixed on the surface of the sensor chip.

BIAevaluation plot of 0.1mM guanine and 0.1mM R-1-P in phosphate buffer 2mM, pH 7.2

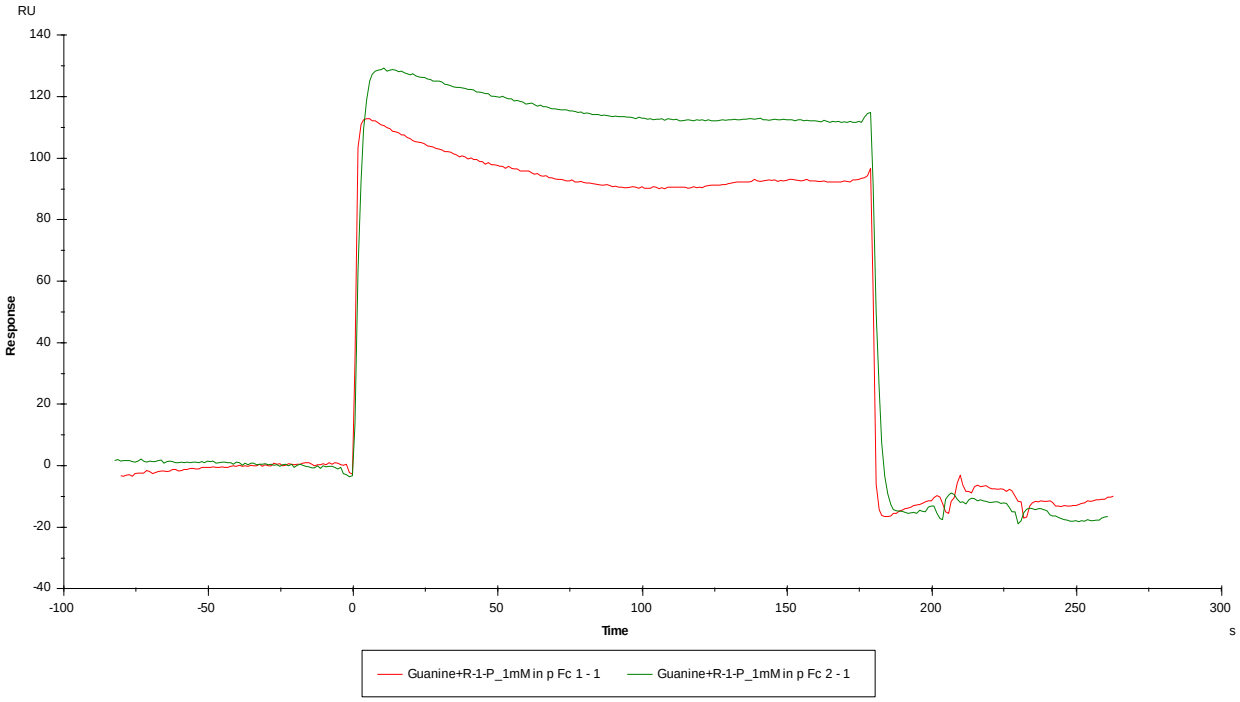


Report point table

Cycle	Fc	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
1	1	104.5	5	14133.9	0.28	-0.11	0.21	Yes	0	baseline
1	2	104.5	5	17079.5	0.39	-0.16	0.28	Yes	0	baseline
1	1	288.5	5	14159.6	4.12	-1.22	3.84	No	25.6	guo+R-P
1	2	288.5	5	17096.7	1.79	0.93	0.53	No	17.1	guo+R-P

Relative response of 0.1 mM solution of guanine and ribose-1-phosphate is rather low. At the beginning of injection there was a decrease in response, but after some time it started to increase. In BIAevaluation plot, the curves were aligned to the same starting point to see that the shape of the curves is quite similar but relative responses vary from each other.

BIAevaluation plot of 1 mM guanine and 1 mM Ribose-1-phosphate in phosphate buffer 2 mM, pH 7.2

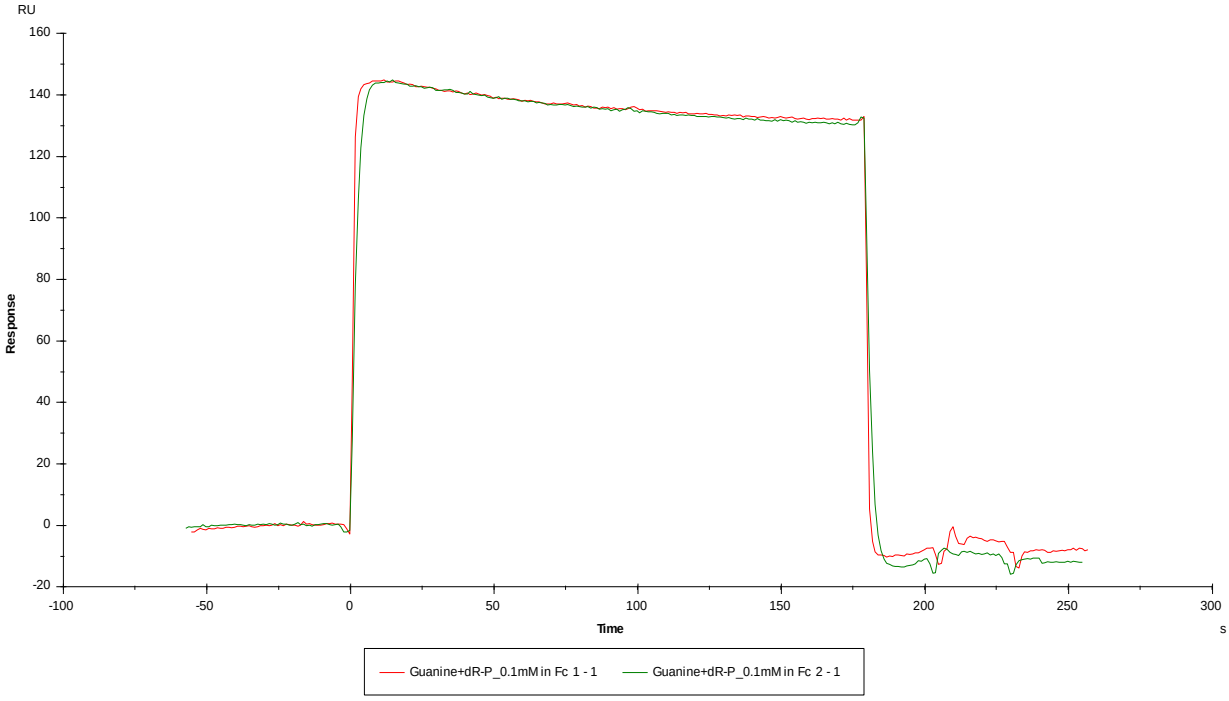


Report point table:

Cycle	Fc	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
1	1	75.5	5	14130.7	0.33	-0.12	0.27	Yes	0	baseline
1	2	75.5	5	17053.8	0.39	-0.17	0.24	Yes	0	baseline
1	1	259.5	5	14201.6	0.81	-17.25	27.93	No	70.9	Guo-R-P
1	2	259.5	5	17167.3	I.47	0.73	0.59	No	113.4	Guo-R-P

In comparison to lower concentration, the relative response of 1 mM solution of guanine and ribose-1-phosphate is severalfold higher. The shape of the curves differs between concentrations but it is same between both (reference and active) cells. Relative response in active sensorgram is higher than that one from reference sensorgram, so there might be some binding anyhow. It is also obvious from relative units in report point table.

BIAevaluation plot of 0.1 mM guanine and 0.1 mM dR-1-P in phosphate buffer 2 mM, pH 7.2

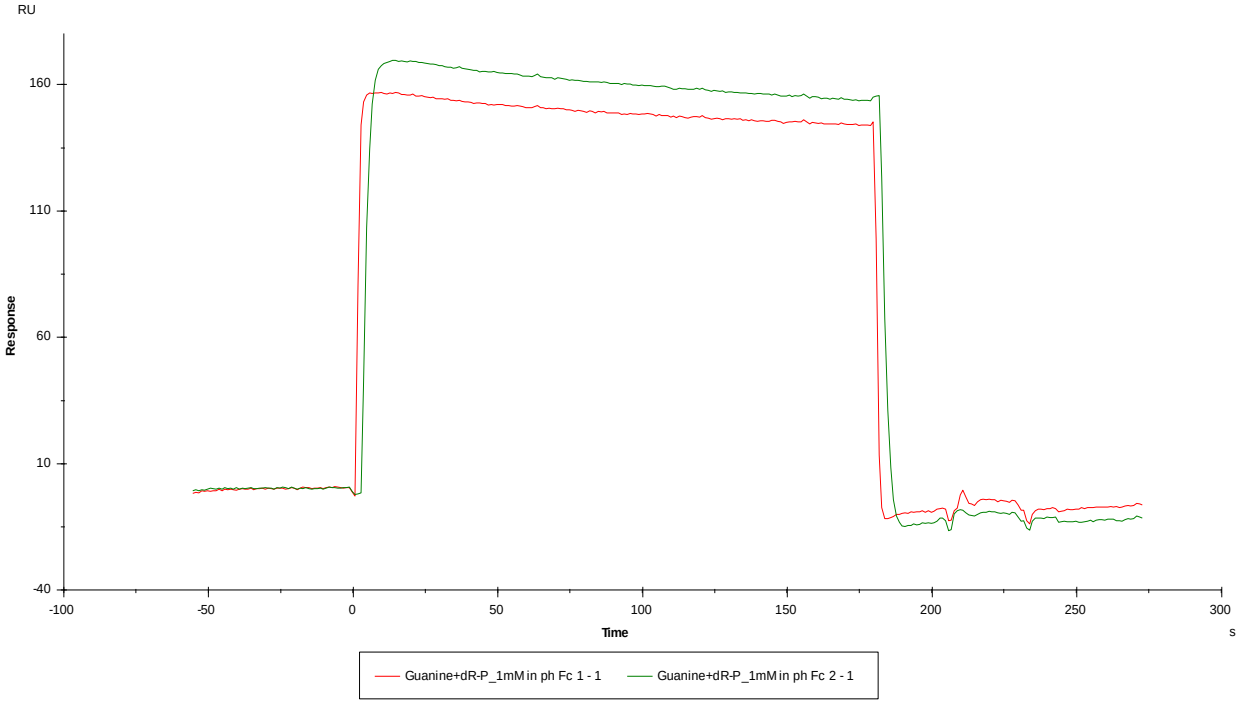


Report point table:

Cycle	Fc	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
1	1	50.5	5.0	14046.4	0.13	-0.05	0.10	Yes	0	baseline
1	2	50.5	5.0	16820.5	0.18	-0.07	0.13	Yes	0	baseline
1	1	234.5	5.0	14148.2	51.83	-22.44	33.98	No	101.8	Guo-dR-P
1	2	234.5	5.0	16951.4	1.16	0.53	0.66	No	130.9	Guo-dR-P

In comparison to the experiments with ribose-1-phosphate, these trials with deoxyribose-1-phosphate do not significantly differ between concentrations as well as between reference and active sensorgrams. The relative response is very high for the concentration of 0.1 mM solution and the shapes of curves are same again. If we compare plots of poor 0.1 mM deoxyribose-1-phosphate with these plots, they are very similar. The shapes are the same, but relative units are lower here.

BIAevaluation plot of 1 mM guanine and 1 mM dR-1-P in phosphate buffer 2 mM, pH 7.2



Report point table:

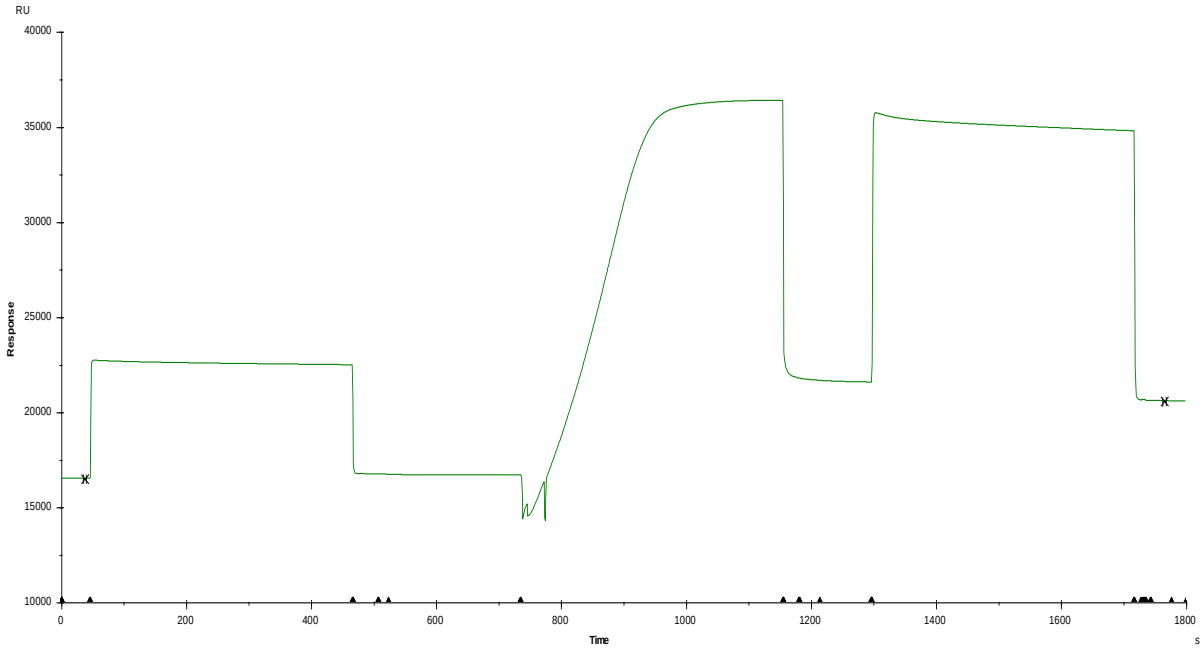
Cycle	Fc	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
1	1	51.5	5.0	14046.3	0.24	-0.06	0.23	Yes	0	baseline
1	2	51.5	5.0	16823.1	0.09	0.02	0.09	Yes	0	baseline
1	1	235.5	5.0	14160.4	52.84	-22.44	35.86	No	114.2	guo-dR-P
1	2	235.5	5.0	16977.0	0.95	0.46	0.47	No	153.9	guo-dR-P

There is almost no difference between 0.1 mM and 1 mM concentration. Relative units are close to those of 0.1 mM guanine and deoxyribose-1-phosphate and also the shape of the curves is same again. But anyhow there is some more specific binding in active cell as it is shown in BIAevaluation plot.

3.2 Results from experiments with TP enzyme

3.2.1 Illustration of immobilizing procedure of TP enzyme

Sensorgram of immobilization of TP enzyme (74,75 µg/ml)



Report point table:

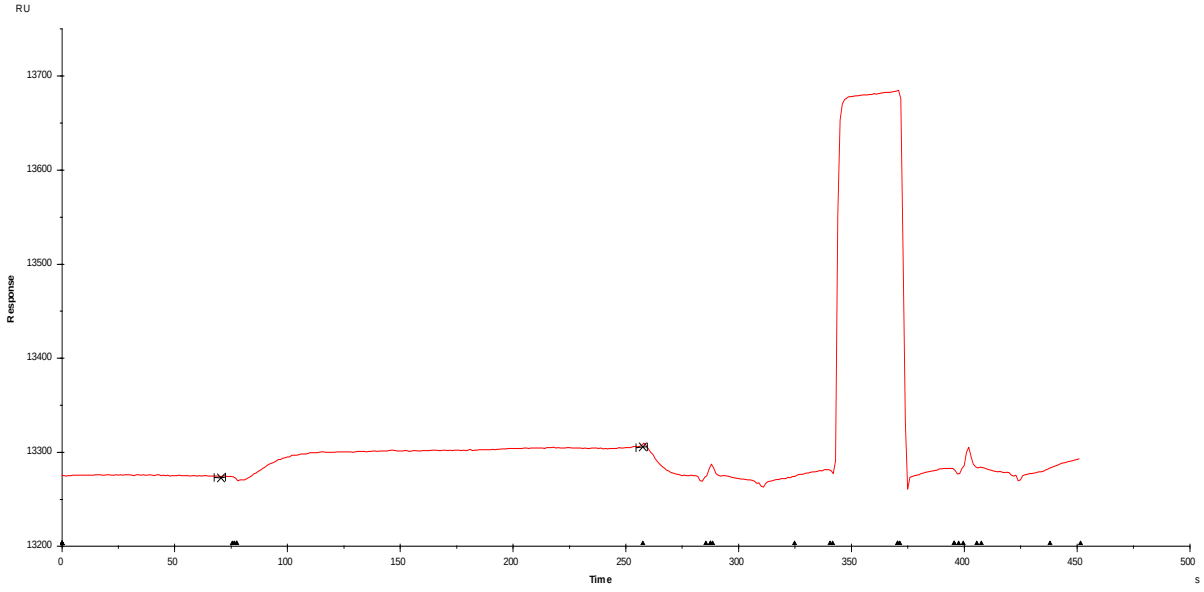
Cycle	Fc	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
1	2	37.5	5.0	16514.9	0.25	0.04	0.26	Yes	0	baseline
1	2	1765.5	5.0	20584.0	1.43	0.58	1.04	No	4069.2	ethanolamine

This is the sensorgram of thymidine phosphorylase immobilizing procedure. The immobilized level of TP enzyme (74,75 µg/ml) is 4069,2 RU. In comparison with PNP enzyme, TP was used in lower concentration, but the immobilized level achieved was much higher.

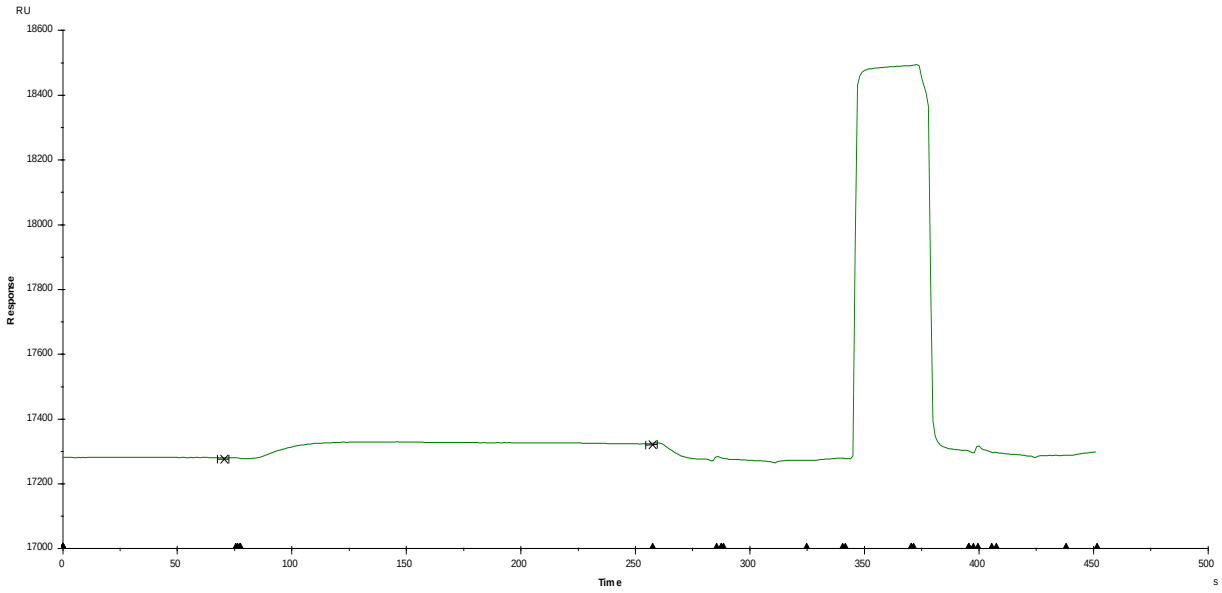
3.2.2 Illustration of interaction analysis between thymidine in solution and TP enzyme immobilized on the surface of the sensor chip.

Sensorgrams of thymidine (0.1mM) dissolved in phosphate buffer (1 mM, pH 7.2), temp. 30ºC

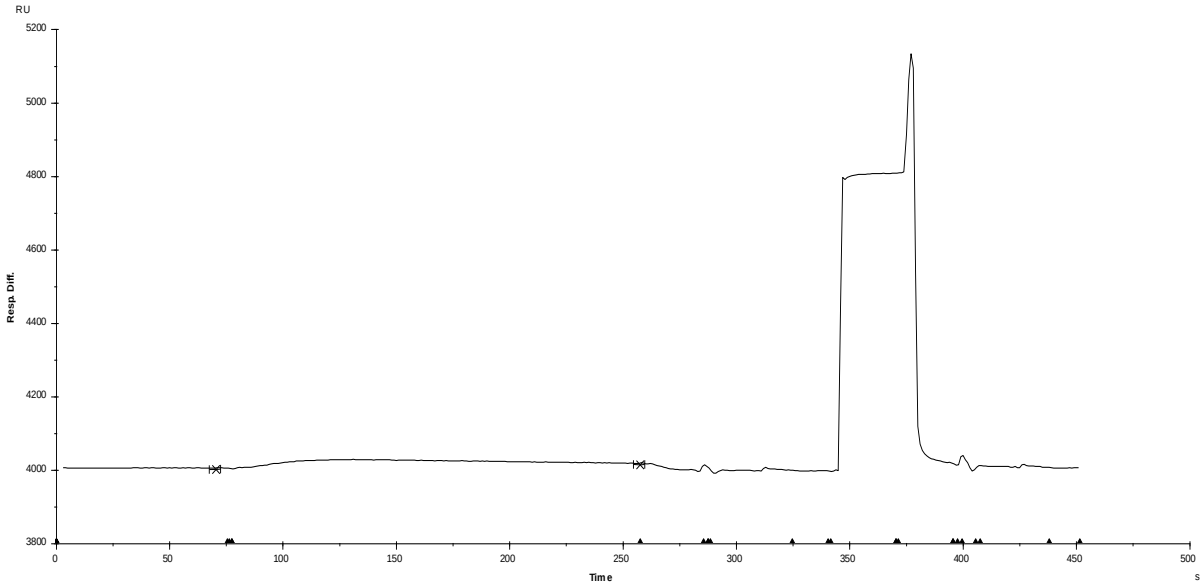
Fc - 1



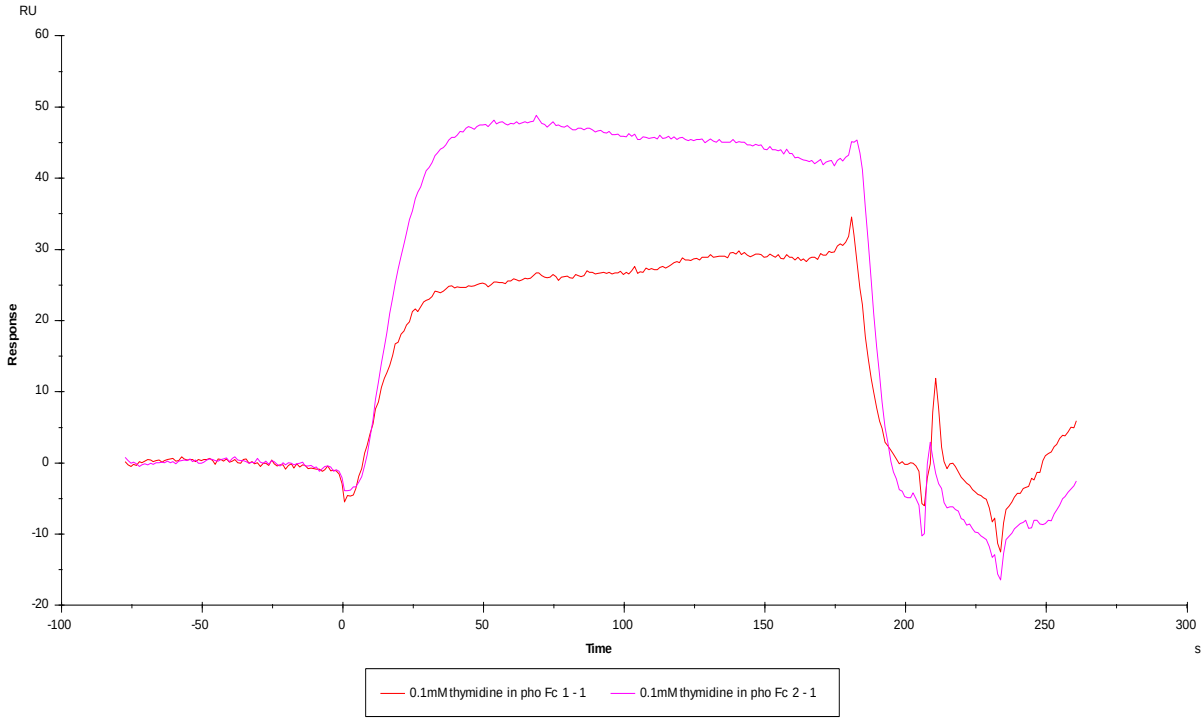
Fc - 2



Fc 2 - 1



BIAevaluation plot of 0.1 mM thymidine in phosphate buffer 1 mM, pH 7.2



There is clearly seen in the BIAevaluation plot, that the binding of thymidine to TP enzyme (Fc 2) exceeded the non-specific binding of thymidine to dextran matrix (Fc 1). The report point table shows the response levels:

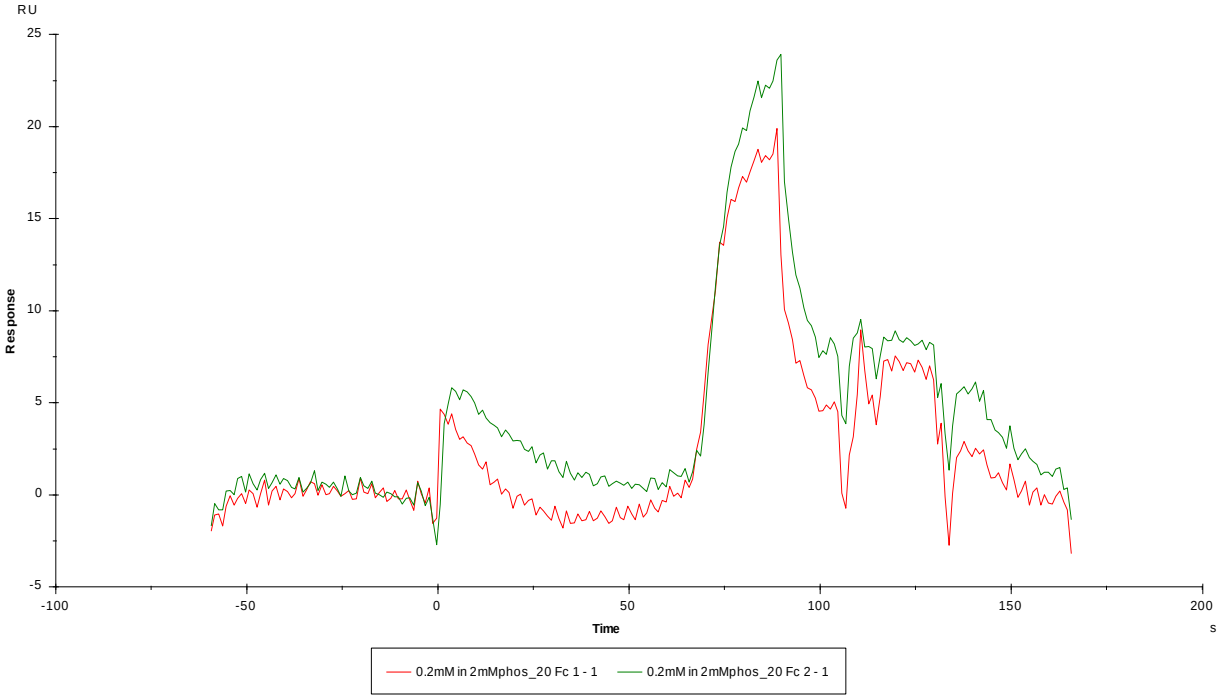
Report point table:

Cycle	Fc	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
1	1	70.5	5	13273.1	0.24	0.04	0.26	Yes	0	baseline
1	2	70.5	5	17276.4	0.27	0.05	0.28	Yes	0	baseline
1	2-1	70.5	5	4003.3	0.36	0.13	0.3	Yes	0	baseline
1	1	257.5	5	13305.8	1.46	0.54	1.19	No	32.6	thymidine
1	2	257.5	5	17320.7	1.2	0.57	0.61	No	44.3	thymidine
1	2-1	257.5	5	4016.1	0.81	0.23	0.77	No	12.8	thymidine

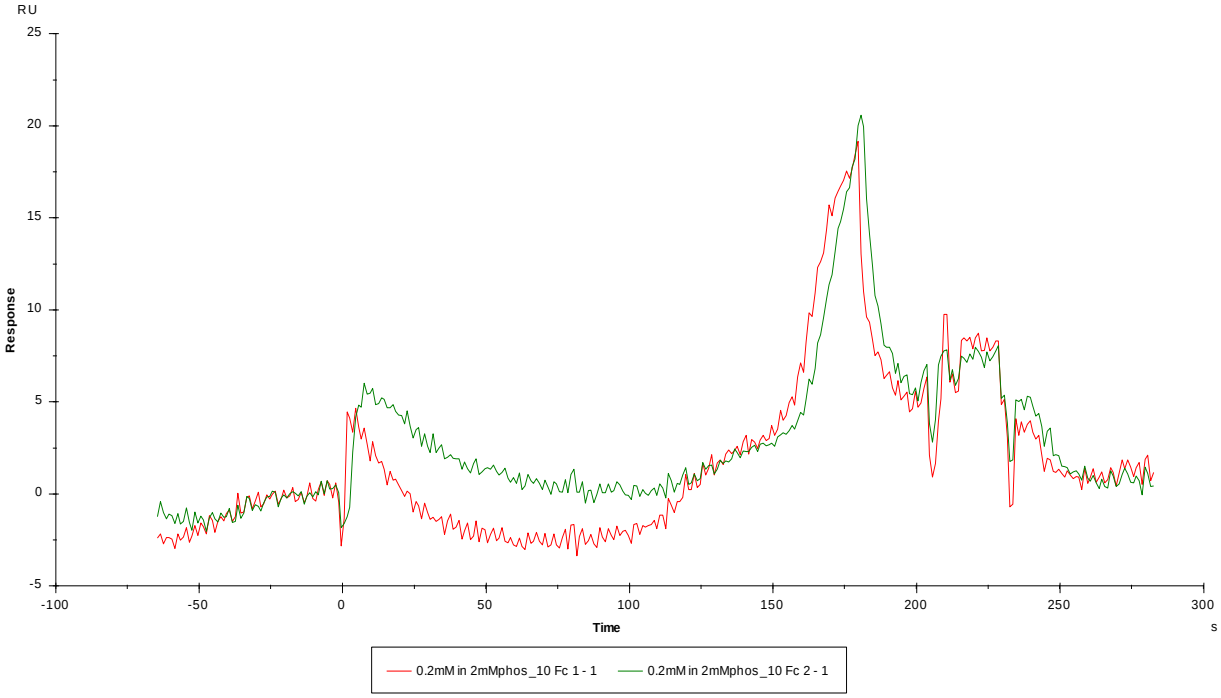
3.2.3 Comparison of BIAevaluation plots from interaction analyses between thymidine and TP enzyme in the usage of different flow rates.

BIAevaluation plots of 0.2 mM thymidine in phosphate buffer 2 mM, pH 7.2

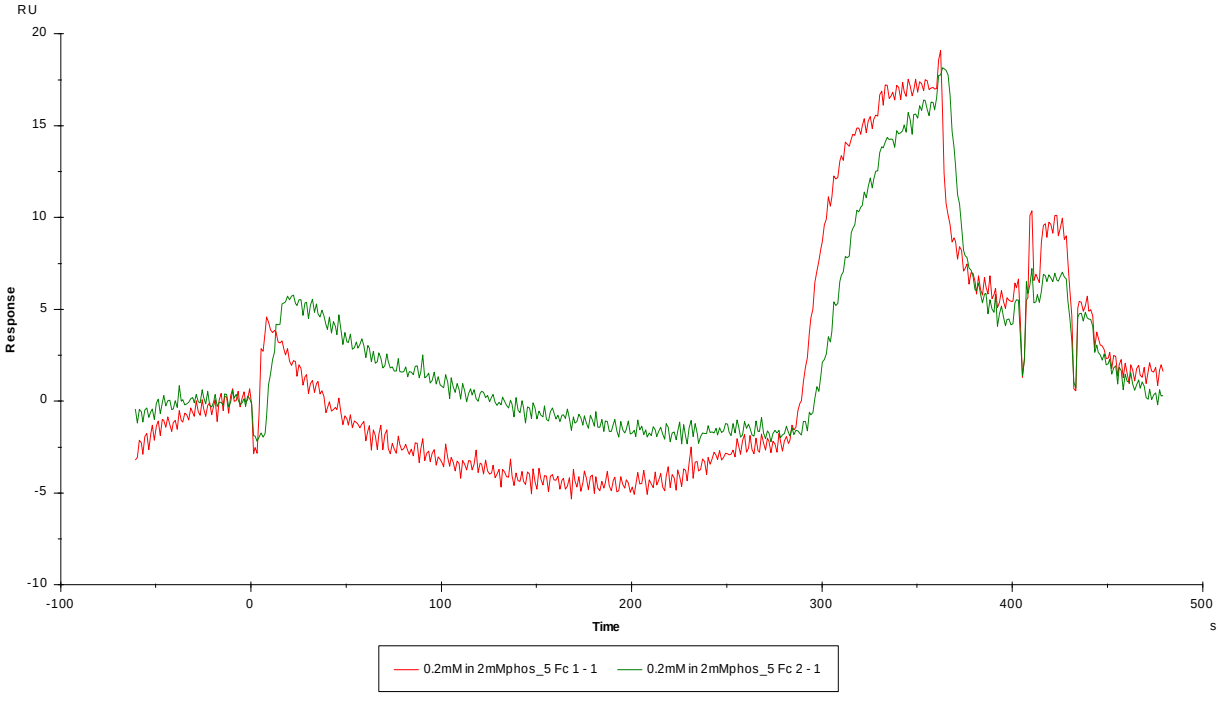
flow rate 20 µl/ml



flow rate 10 µl/ml



flow rate 5 µl/ml



Report point tables:

thymidine 0.2 mM in phosp.buffer 2 mM, flow rate 20 µl/ml

Cycle	Fc	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
1	1	53.5	5	13863.7	0.54	0.07	0.59	Yes	0	baseline
1	2	53.5	5	17912.5	0.45	0.16	0.38	Yes	0	baseline
1	1	147.5	5	13881.5	2.37	-0.58	2.36	No	17.7	thymidine
1	2	147.5	5	17935.3	0.92	0.46	0.34	No	22.7	thymidine

thymidine 0.2mM in phosp.buffer 2mM, flow rate 10 µl/ml

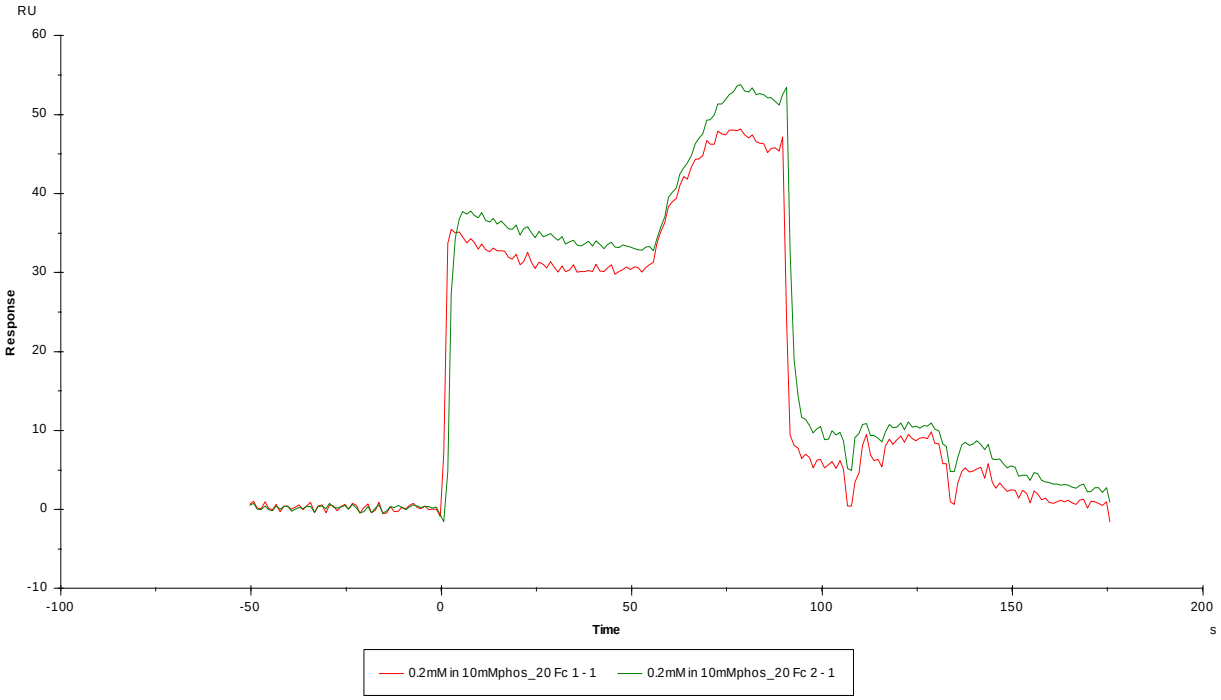
Cycle	Fc	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
1	1	52.5	5	13862.8	0.38	0.06	0.4	Yes	0	baseline
1	2	52.5	5	17918	0.24	0	0.27	Yes	0	baseline
1	1	244.5	5	13878.9	3.27	-1.25	2.56	No	16.1	thymidine
1	2	244.5	5	17937	1.56	0.77	0.67	No	19	thymidine

thymidine 0.2mM in phosp.buffer 2mM, flow rate 5 µl/ml

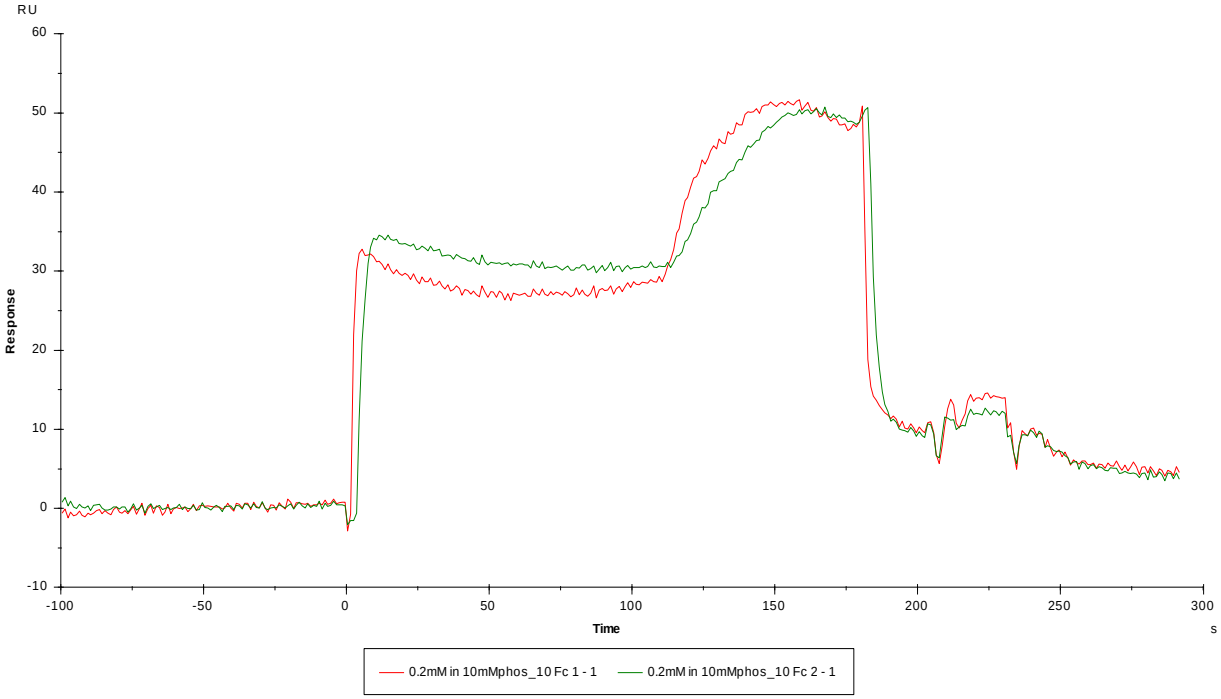
Cycle	Fc	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
1	1	56.5	5	13862.2	0.33	0	0.37	Yes	0	baseline
1	2	56.5	5	17916.5	0.24	-0.08	0.21	Yes	0	baseline
1	1	422.5	5	13879.5	1.22	0.03	1.37	No	17.3	thymidine
1	2	422.5	5	17933.6	0.96	0.47	0.41	No	17	thymidine

BIAevaluation plots of 0.2 mM thymidine in phosphate buffer 10 mM, pH 7.2

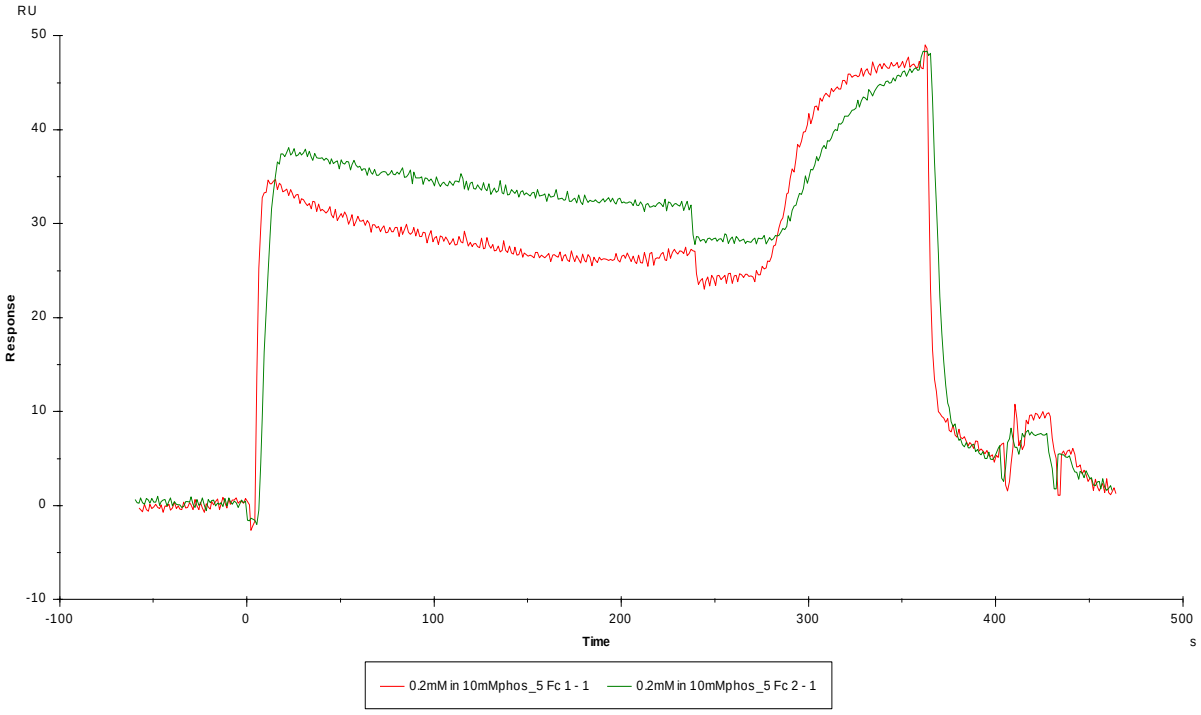
flow rate 20 µl/ml



flow rate 10 µl/ml



flow rate 5 µl/ml



Report point tables:

thymidine 0.2 mM in phosp.buffer 10 mM, flow rate 20 µl/ml

Cycle	Fc	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
1	1	96.5	5	14024.9	0.29	-0.11	0.23	Yes	0	baseline
1	2	96.5	5	18110.5	0.13	-0.01	0.14	Yes	0	baseline
1	1	191.5	5	14060.7	15.85	-7.01	9.95	No	35.9	thymidine
1	2	191.5	5	18159	7.95	-2.57	7.08	No	48.6	thymidine

thymidine 0.2 mM in phosp.buffer 10 mM, flow rate 5 µl/ml

Cycle	Fc	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
1	1	50.5	5	14026.1	0.41	0.15	0.35	Yes	0	baseline
1	2	50.5	5	18113.2	0.39	0.03	0.43	Yes	0	baseline
1	1	421.5	5	14067.2	10.28	-4.29	7.18	No	41.2	thymidine
1	2	421.5	5	18160.5	0.97	0.47	0.45	No	47.2	thymidine

thymidine 0.2 mM in phosp.buffer 10 mM, flow rate 10 µl/ml

Cycle	Fc	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
1	1	93.5	5	14023	0.3	0.08	0.29	Yes	0	baseline
1	2	93.5	5	18110.8	0.38	0.02	0.43	Yes	0	baseline
1	1	280.5	5	14064	12.58	-5.31	8.63	No	41	thymidine
1	2	280.5	5	18159.8	0.89	0.45	0.35	No	49	thymidine

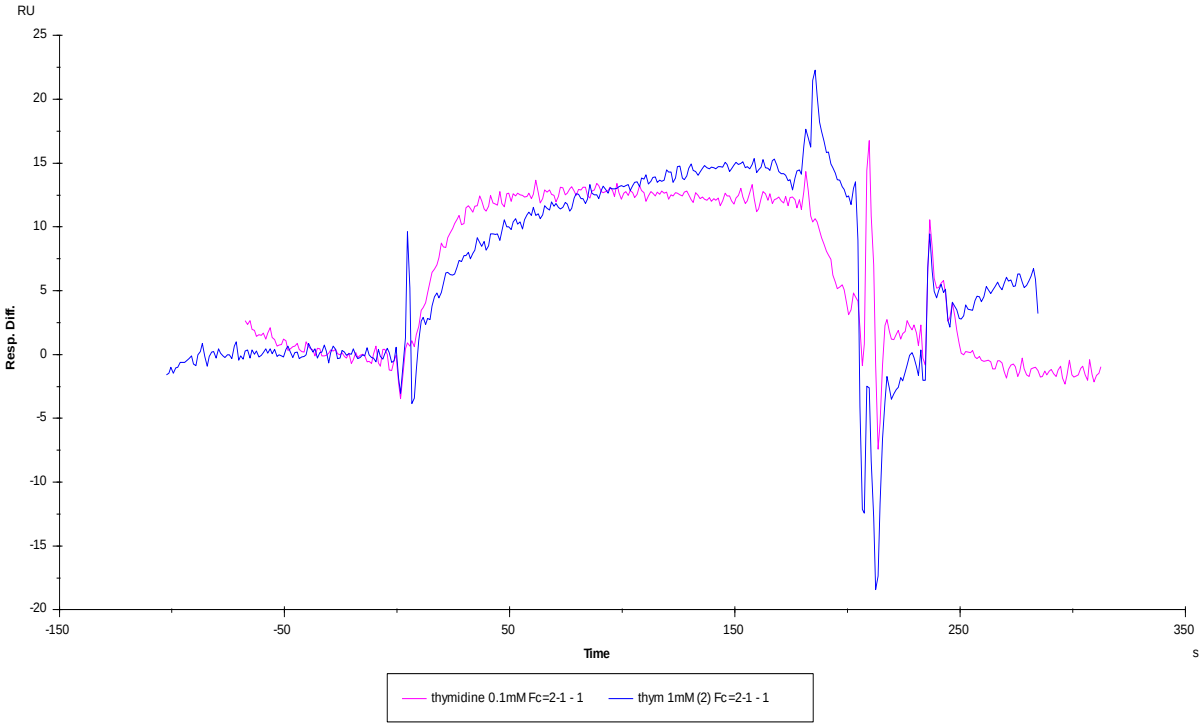
These BIAevaluation graphs above compare binding of 0.2 mM thymidine, dissolved in 2 mM and 10 mM phosphate buffer, to TP enzyme with reference surface at three different flow rates. The response from reference cell (red) usually copies the curve from active cell (green) or vice versa. There are no significant differences in binding between the cells.

The flow rate influences the time, in which the substrate is in contact with the surface of the chip, and subsequently with enzyme. But it has no influence on binding of substrate to enzyme.

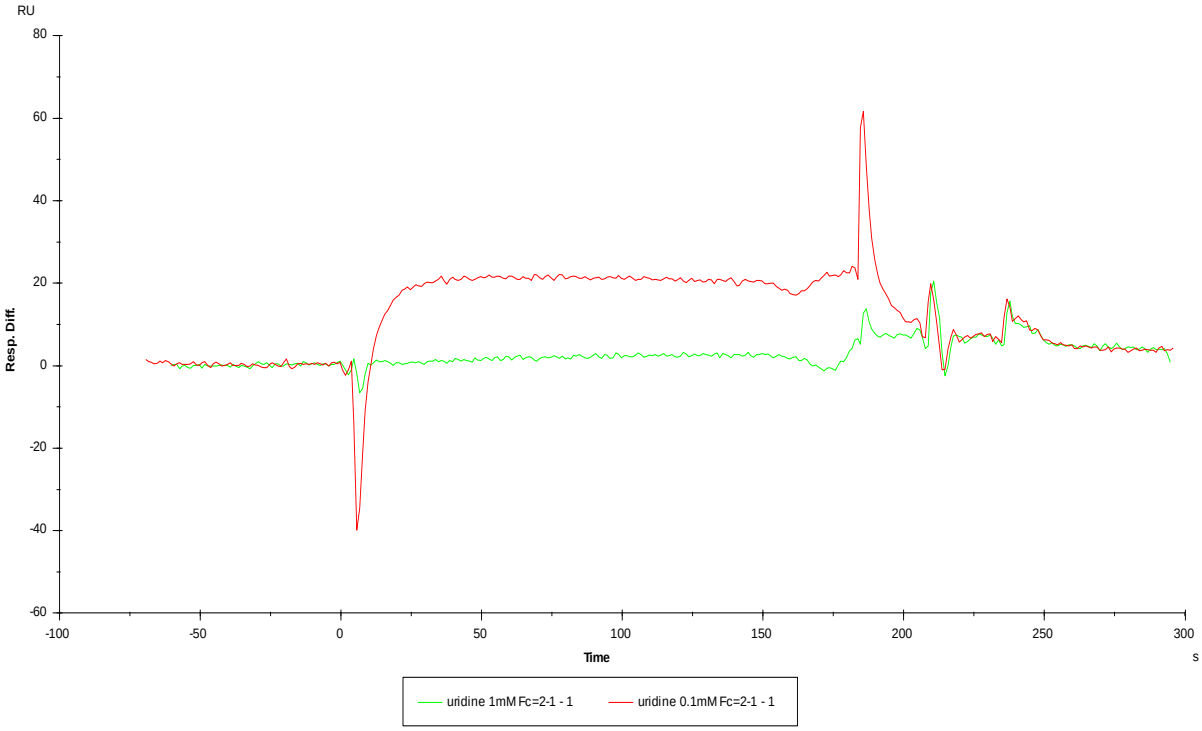
Visible differences are between two molarities of phosphate buffers. The more concentrated phosphate buffer gives higher relative responses (in both of the cells) than the more diluted phosphate buffer.

3.2.4 Comparison of BIAevaluation plots from interaction analyses between thymidine or uridine or deoxy-uridine and TP enzyme.

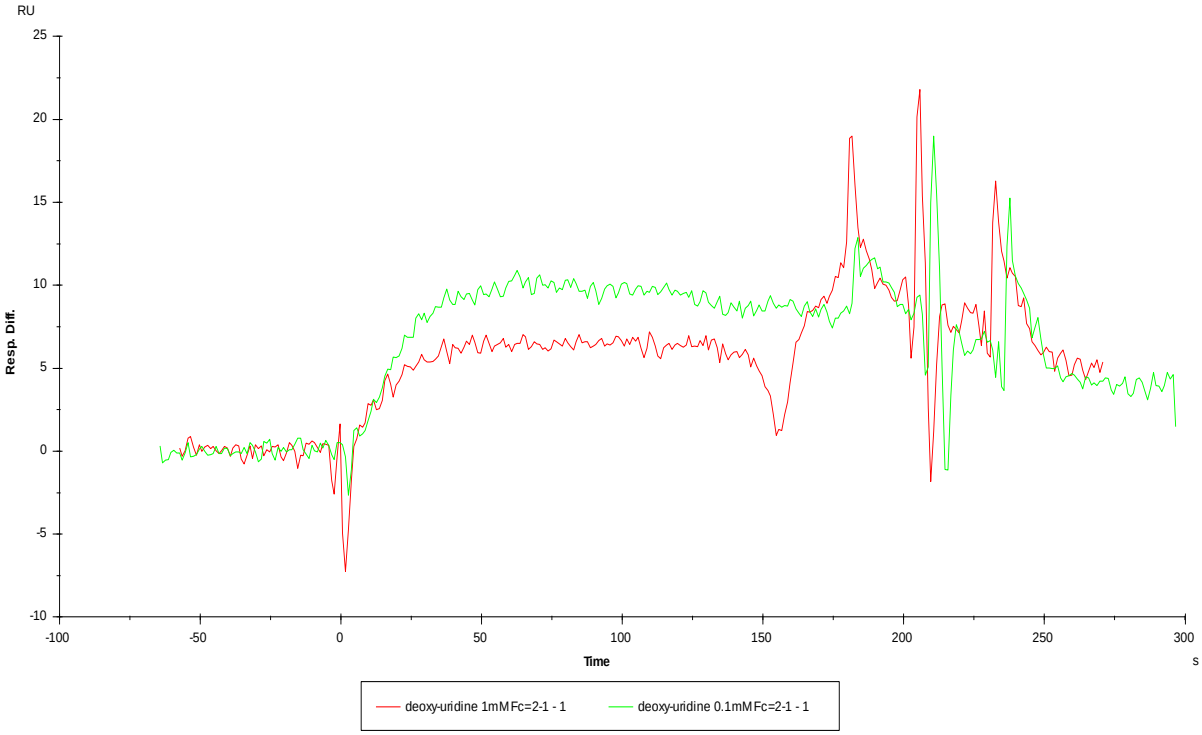
BIAevaluation plot comparing two concentrations of thymidine (0,1 mM and 1 mM)



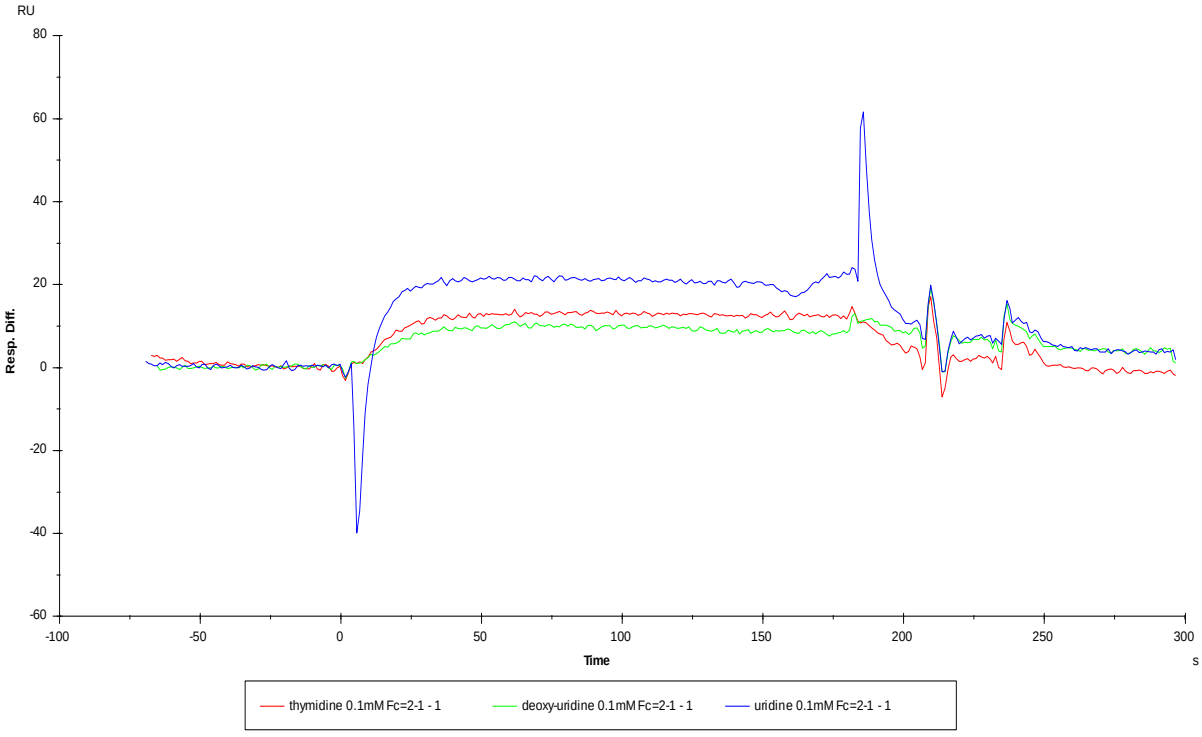
BIAevaluation plot comparing two concentrations of uridine (0,1 mM and 1 mM)



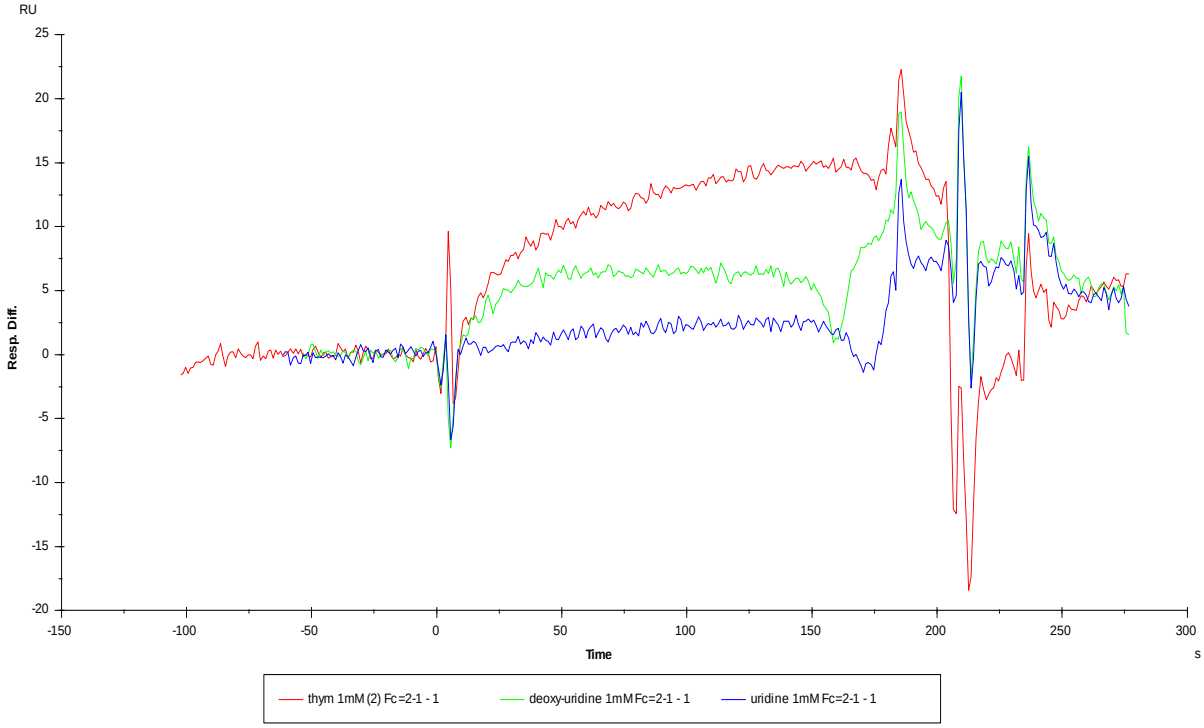
BIAevaluation plot comparing two concentrations of deoxy-uridine (0,1 mM and 1 mM)



BIAevaluation plot comparing thymidine, uridine and deoxy-uridine in concentration 0,1mM



BIAevaluation plot comparing thymidine, uridine and deoxy-uridine in concentration 1mM



The first part of BIAevaluation plots compare the same substrate of two different concentrations and the other part compare three different substrates of the same concentration. It is interesting, that lower concentration of the substrate gives better responses than higher concentration, except for thymidine. This event is possible to see also in the last plot, where thymidine of higher concentration provides the best response from all of substrates.

4. DISCUSSION

In this study I was testing the binding of natural substrates to their enzymes, using the different conditions. PNP enzyme was the main object of the investigation, but the results were not as satisfactory as was expected, then TP enzyme was also tested.

PNP enzyme

At the beginning, a convenient concentration of PNP enzyme was sought for the coating of the sensor chip. It is obvious from the results that with the rising concentration of enzyme the immobilized level did not increase proportionally. The best concentration of PNP enzyme for immobilization procedure was 300 μg/ml.

The conditions during the interaction analyses were changed to find out the best environment for the binding of substrates to enzyme. As a natural substrate for PNP enzyme we tested mainly guanosine (participating in the way of cleavage), dissolved in various concentrations and pH of buffer. In comparison of pH of buffers, none of the pH values showed noticeable preferences. In comparison of types of buffers, better results were achieved with phosphate buffer than with the HBS-EP buffer.

As other natural substrates we used guanine and at the end also ribose-1-phosphate and deoxyribose-1-phosphate.While guanosine was easily dissolved, guanine wasn't. It is difficult to estimate how much of weighed amount of guanine was in testing solution, but some changes in the response occurred, which means that some guanine has bound to enzyme. For better dissolving of guanine an organic solvent DMSO was used, but its background response was so great, that it covered the sensitive binding of guanine and it didn't even help with solubility.

TP enzyme

While testing TP enzyme, usually two concentrations of substrate solutions were examined and compared in changing conditions like type and molality of buffer, which was used as solvent and running medium, then temperature and flow rate.

A great difference appeared in sensorgram when running buffer was dissimilar to that used as a solvent for substrate. It caused so big fall in response, that any interaction between enzyme and substrate couldn't be seen. Concentration of buffer also influenced the response. I used mostly phosphate buffer and phosphate ions participate in reaction catalyzed by TP. Higher molarity of buffer usually, but not every time, caused bulk shift in response level. The best results were obtained when the 1mM phosphate buffer and 0.1 mM solution of thymidine were used. The reason could be the high sensitivity of instrument Biacore X, because then small nuances are more visible than at higher concentrations, where the phosphate ions "cover" the binding process.

Increase of temperature didn't improve enzyme activity in binding with substrate; it only caused undesirable air bubbles in the system. Finally, neither the change of flow rate had any effect on substrate-enzyme interaction. Although the flow rate determines the time period, during which the substrate is in contact with enzyme, it had no impact on binding curves.

5. CONCLUSION

In this study, the ligand binding properties of two enzymes – purine nucleoside phosphorylase and thymidine phosphorylase – were tested, exploiting a method of surface plasmon resonance in Biacore X instrument.

PNP is a ubiquitous enzyme playing a key role in the purine salvage pathway and TP plays an important role in human organism too, especially in pathological processes like inflammation or cancer. Both of enzymes are potential tools for the enzymatic synthesis of nucleoside analogues, which may possibly be used as antiviral or anticancer agents and that are difficult to prepare by chemical synthesis, or are obtained in a low yields.

Use of Biacore X instrument helps to reveal how the enzymes interact with their natural substrates or derivatives. This work was focused on searching for the most suitable conditions, at first for immobilizing procedure (coating the surface of the sensor chip with PNP and TP enzyme) and then for interaction analysis between the enzymes and their natural substrates. The present results can be used as starting point for additional

investigations of interaction analyses between enzymes and semi-synthetic analogues or they can also contribute to optimize conditions of producing such compounds by biocatalysis. In particular, the use of enzymes as biocatalysts allow the stereo- and regio-selective formation of the glycosidic bond and application of this technique may improve the availability of desired nucleosides.

6. REFERENCES

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7. SUMMARY IN CZECH (SOUHRN PRÁCE)

Tato práce se zabývá zkoumáním vazebných vlastností a interakcí u enzymů purinnukleosidfosforylasa a thymidinfosforylasa s jejich přirozenými substráty s využitím metody SPR analýzy v přístroji Biacore X.

Cílem práce bylo nejprve zjistit, zda a jak mohou být enzymy, připravené technologií rekombinantní DNA, ukotveny na povrch analytického čipu CM 5 s volnými karboxylovými skupinami. Poté bylo testováno, jakým způsobem a za jakých podmínek se již ukotvené enzymy vážou se svými přirozenými substráty. Měnícími se podmínkami byly druh pufru, jeho pH a molalita, teplota v systému a průtoková rychlost celou.

Jako přirozené substráty pro enzym PNP byly použity guanosin ve směru rozkladné reakce a guanin s ribóza-1-fosfát nebo s deoxyribóza-1-fosfát ve směru syntézy. Pro enzym TP, který byl studován pouze ve směru fosforolýzy, byly jako přirozené substráty použity thymidin, uridin, a deoxyuridin.

V závěru byla opakována interakční analýza k ověření stability čipu pokrytého TP enzymem, který byl uskladněn v lednici po dobu 10 týdnů.

Přístroj Biacore X je založen na metodě SPR analýzy. Surface plasmon resonance neboli povrchová plasmonová rezonance je neinvazivní optická metoda sloužící ke sledování interakcí mezi biomolekulami a jejich ligandy. Odpověď nezávisí na povaze biomolekuly, proto přístroj umožňuje měřit široké spektrum vzorků – od vysoce čistých molekul po hrubé vzorky jako jakou viry, bakterie či eukaryotické buňky. Kromě klasického analytického stanovení koncentrací analytů lze získat též časový průběh reakce interakcí biomolekul, provádět kinetické studie a určovat kinetické parametry pro sledované děje.

Povrchová plasmonová resonance je jev vyskytující se v tenkém vodivém filmu na rozhraní dvou medií s odlišným refraktivním indexem. V systému Biacore jsou těmito médii sklo senzorového čipu a roztok vzorku. Vodivým filmem je tenká vrstva zlata na povrchu čipu. V určité kombinaci úhlu dopadu a vlnové délky dopadajícího světla dochází k excitaci plasmonů z vrstvy zlata. Přitom dochází k absorpci energie, což se projevuje snížením intenzity odraženého světla. Změny v koncentraci roztoku na

povrchu čipu způsobují změny v SPR signálu. Ten je vyjádřen v resonančních jednotkách RU, kde jedna RU odpovídá jednomu pikogramu na čtverečný milimetr povrchu čipu.

Pro imobilizaci enzymu PNP na povrch senzorového čipu byly testovány tři různé koncentrace - 100μg/ml, 300μg/ml a 600μg/ml. Ze získaných výsledků vyplývá, že stupeň imobilizace enzymu neodpovídá úměrně vzrůstající koncentraci roztoku a jako nejvhodnější byla stanovena koncentrace enzymu 300μg/ml. Imobilizace enzymu TP probíhala ve srovnání s PNP snadněji. Již relativně nízké koncentrace enzymu v roztoku (74,75μg/ml) poskytly mnohem vyšší stupeň imobilizace.

Při hledání optimálních podmínek pro interakci enzym – substrát byly měněny tyto parametry: teplota celého systému, průtoková rychlost celou a zejména pufr – druh, jeho pH a molalita. Zvýšení teploty nemělo významný vliv na vazebné vlastnosti enzymů ani substrátů; naopak mělo za následek tvorbu nežádoucích vzduchových bublin. Zpomalením anebo zrychlením průtoku mobilní fáze s roztokem vzorku detekční celou se vazebné vlastnosti opět nijak výrazně nezměnily. Rychlost pouze ovlivnila dobu, po kterou byl analyt v kontaktu s enzymem, což se projevilo na „šířce“ grafu, ale tvar křivky ovlivněn nebyl.

Pufr měl v systému dvě funkce. Sloužil jako mobilní fáze a zároveň jako rozpouštědlo pro vzorky analytů. Využívala jsem převážně dva druhy fosfátových pufrů – HBS-EP pufr od výrobce a mnou připravený fosfátový pufr. Při použití HBS-EP pufru jako mobilní fáze a fosfátového pufru jako rozpouštědla pro vzorek došlo k silné odpovědi a výraznému poklesu signálu, tudíž jakékoliv vazebné interakce nemohly být vyhodnoceny. Molalita pufru se osvědčila spíše nižší a pH pufru kolem 7, tedy neutrální.

Při ověřování stability čipu pokrytého enzymem po určité době uchovávání v chladu byla zjištěna velmi zeslabená účinnost enzymu.

Oba enzymy, purinnukleosidfosforylasa i thymidinfosforylasa, hrají v organismu důležitou roli. PNP je klíčovým enzymem v metabolismu purinů, zejména v jejich záchranných procesech a TP je významným enzymem patologických procesů jako je zánět či rakovina. Podrobné zkoumání těchto enzymů přispívá k jejich využití jako biokatalyzátorů pro produkci semi-syntetických purinových či pyrimidinových

nukleosidových analogů, které mohou být použity jako potenciální antivirotika či kancerostatika. Tyto se obtížně získávají organickou syntézou, která je časově i finančně náročná, s nízkými výtěžky a bez stereospecifity produktů.

Tato práce je součástí výzkumného projektu „Biocatalysts for producing pharmaceutical nucleoside analogues“ / „Biokatalyzátory pro produkci farmaceutických nukleosidových analogů“, kterého se účastní kromě University of Kuopio též University of Helsinky a University of Oulu.