Department of Physics, Chemistry and Biology

Bachelor's Thesis

In vitro studies of Thiopurine S-Methyltransferase:
Ligand binding interactions and development of a new enzymatic
activity assay for TPMTwt, TPMT*6 and TPMT*8

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Linköping University INSTITUTE OF TECHNOLOGY
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In this thesis we have investigated how the wild type and two variants of TPMT interact with different ligands using fluorescence and isothermal titration calorimetry. Experiments with MTX, ANS and furosemide resulted in a similar binding strength for the wild type and the variant TPMT*8, while the other variant TPMT*6 showed a slightly weaker binding. A binding affinity for polyglutamated MTX to TPMTwt was also determined which resulted in an almost twice as strong binding compared to MTX.

Today’s methods to determine enzymatic activity are either based on radioactivity, time consuming or expensive. As an alternative the use of a spectrophotometric assay using 5-thio-2-nitrobenzoic acid (TNB) was investigated. The method showed positive results and could hopefully be adapted to plate readers in future experiments. Using 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB, also known as Ellman’s reagent) the amount of accessible thiol groups on the protein was estimated. This revealed a similar relationship between TPMTwt and TPMT*6, while the result for TPMT*8 was inconclusive.

Keyword
Thiopurine S-Methyltransferase; 6-mercaptopurine; methotrexate; isothermal titration calorimetry; fluorescence spectroscopy
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Linköpings University
2015

Figure 1: Structure of human Thiopurine S-Methyltransferase. Drawn from 2BZG.pdb in PyMol.
Abstract

Acute lymphoblastic leukemia, one of the most malignant cancer forms in children is commonly treated with the thiopurine 6-mercaptopurine (6-MP) in combination with a high dose of methotrexate (MTX). 6-Mercaptopurine is in the body metabolized by the enzyme thiopurine S-methyltransferase (TPMT). Polymorphic variants of TPMT express different catalytic activities, and for this reason the dosage of 6-MP needs to be individualized. In order to better optimize the treatment it is important to understand how mutations in TPMT affect its enzymatic activity.

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I det här arbetet har vi undersökt hur vildtypen och två varianter av TPMT interagerar med olika ligander med hjälp av fluorescens och isotermisk titrerings kalorimetri. Mätningar med MTX, ANS och furosemid resulterade i en liknande bindningsstyrka för vildtypen och variant *8 medan variant *6 gav en något svagare bindning. En bindningsaffinitet för polyglutamerat MTX till vildtypen har också bestämts vilket resulterade i en nästan dubbelt så stark bindning jämfört med MTX.

Dagens metoder för att bestämma enzymaktivitet är antingen baserade på radioaktivitet eller så kräver dem mycket tid eller pengar. Som ett alternativ testade vi att använda en spektrofotometrisk metod med tillsats av 5-tio-2-nitrobensoesyra (TNB) som substrat. Detta gav fina resultat och vår förhoppning är att denna metod även i framtiden skulle kunna appliceras på plattläsare.

Genom att använda 5,5′- ditiobis-2-nitrobensoesyra (DTNB; också känd som Ellmans reagens) kunde vi uppskatta antalet exponerade tiolgrupper på proteinet. Detta resulterade i en liknande relation mellan vildtypen och TPMT*6 medan resultatet för TPMT*8 var ofullständigt.
## Abbreviations

<table>
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<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>6-MP</td>
<td>6-mercaptopurine</td>
</tr>
<tr>
<td>6-TG</td>
<td>6-thioguanine</td>
</tr>
<tr>
<td>6-TGNs</td>
<td>6-thioguanine nucleotides</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>ANS</td>
<td>8-anilinonaphthalene-1-sulfonic acid</td>
</tr>
<tr>
<td>AZA</td>
<td>Azathioprine</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolate reductase</td>
</tr>
<tr>
<td>DNPS</td>
<td>De novo purine synthesis</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman’s reagent)</td>
</tr>
<tr>
<td>HGPRT</td>
<td>Hypoxanthine guanine phosphoribosyl transferase</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>MTXPG</td>
<td>Methotrexate polyglutamate</td>
</tr>
<tr>
<td>SAH</td>
<td>S-adenosylhomocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine hydrochloride</td>
</tr>
<tr>
<td>TIMP</td>
<td>Thioinosine monophosphate</td>
</tr>
<tr>
<td>TNB</td>
<td>5-thio-2-nitrobenzoic acid</td>
</tr>
<tr>
<td>TPMT</td>
<td>Thiopurine S-methyltransferase</td>
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Introduction

Thiopurines are commonly used drugs in treatment of acute lymphoblastic leukemia (ALL), inflammatory bowel disease (IBD) and autoimmune diseases. The metabolism of thiopurines is catalyzed by thiopurine S-methyltransferase (TPMT). This is a pharmacogenetic enzyme whose genetic polymorphism is important for achieving an optimal dose of thiopurines. TPMT converts prodrugs like azathioprine (AZA) and 6-mercaptopurine (6-MP) to 6-thioguanine nucleotides (6-TGNs). 6-TGNs are then incorporated into DNA and induce apoptosis (Wennerstrand P., 2012).

Acute lymphoblastic leukemia is one of the most malignant cancer diseases among children. The treatment of ALL includes a high dose of methotrexate (MTX) in combination with 6-MP. Patients who undergo this kind of treatment have to get their blood analyzed to determine their TPMT activity. The dose of thiopurines is then individualized in order to optimize the treatment and to avoid life-threatening side effects (Wennerstrand P., 2012).

MTX and 6-MP both produce remission in ALL but the combination raises the children's survival remarkably. Both MTX and 6-MP interact with important pathways like methylation reactions and purine synthesis. They inhibit the de novo purine synthesis (DNPS) alone whereas 6-MP plus high dose MTX produce full inhibition in vivo. However, the exact biochemical mechanism of their synergism is still unknown (Dervieux T. et al., 2002; Giverhaug T., Loennechen T., Aarbakke J. 1999).

The interaction of recombinant TPMT with drugs has previously been investigated by different research groups. Fluorescence spectroscopy and isothermal titration calorimetry (ITC) are two techniques widely used in ligand binding studies. The most common method, fluorescence spectroscopy has revealed successful binding to both MTX and 8-anilinonaphtalene-1-sulfonic acid (ANS) (Wennerstrand P., 2012). Most of the studies in vitro are performed on the wild type and further binding experiments with different TPMT variants are therefore of high interest.

In this thesis, interaction of TPMT*6 and TPMT*8 with ANS, MTX and furosemide was investigated using fluorescence spectroscopy and ITC. In addition the binding affinity for polyglutamated MTX to TPMTwt was measured in order to determine this process’s effect on the binding strength.

Radiochemical assays have been useful methods for monitoring the enzyme activity in vivo. Absorbance measurements at 320 nm with 6-MP as substrate has previously been performed in vitro (Wennerstrand P., 2012). Both of these methods are time consuming yet new and faster methods like SAMfluoro™ are quite expensive. A quick and cheap spectrophotometric assay for determining the catalytic activity of recombinant TPMT will therefore be investigated using 5-thio-2-nitrobenzoic acid (TNB) as a substrate.

Our thesis will also include optimization of a method for detecting thiol-disulfide exchange, which in turn can give an indication of the enzyme's stability. The results and optimization of methods can hopefully be used in further research within the field of thiopurine treatments.
**Aim of the project**

The purpose of this thesis was to investigate how properties of selected TPMT variants differ *in vitro*. This was done by measuring the binding affinity for MTX, ANS and furosemide to each of the variants. A study on how polyglutamation affects MTX binding was also performed on TPMTwt. Apart from binding studies we also developed a new spectrophotometric method to determine TPMT’s enzymatic activity. Further, using 5,5'-dithiobis-(2-nitrobenzoic acid) (known as DTNB or Ellman’s reagent) we also investigated whether changes in the variants structure could cause formation of new disulfide bonds.
Theoretical background TPMT and ligands

History

The enzyme was found in mammalian tissue in 1963, twenty years before it was identified in human kidney in 1983. The enzyme's role in the metabolism of thiopurine drugs has been known since the eighties when it was discovered that patients with mutations in the TPMT gene obtained severe side effects after treatment with 6-MP (Mulder D. J. et al., 2014).

6-mercaptopurine was tested in childhood leukemia already in 1953, 30 years before the discovery of human TPMT (Burchnal J. H. et al., 1953). A man named George Hitchings proposed a theory that non-functioning analogues of nucleic acid bases could be used to stop cell growth. Hitchings and his colleagues substituted an atom at the 6-position of guanine and proved the cytotoxic effect of 6-MP and 6-TG in leukemia cells. George Hitchings and Gertrude Elion were awarded the Nobel Prize in Medicine in 1988.

TPMT structure and properties

TPMT is a single-domain monomeric protein composed of 245 amino acids. It belongs to class I of SAM dependent methyltransferases. The structure shows a typical Rossman fold which consists of a nucleotide-binding domain. TPMT contains open twisted β-sheets surrounded by α-helices on both sides creating a topological switch where TPMT binds S-adenosylmethionine (SAM) (Schubert H. L. et al., 2003). SAM is the second most widely used substrate after ATP. It is responsible for donating methyl groups to the substrate which results in the co-product S-adenosylhomocysteine (SAH). Unfortunately TPMT’s natural substrate is still unknown. The three-dimensional structure of truncated human TPMT bound to SAM has been determined to high resolution (Figure 2). The apo form of human TPMT has not been identified because of its flexibility in the active site (Wu H. et al., 2007).

Genetic polymorphism of TPMT

TPMT is a pharmacogenetic enzyme whose genetics is important for achieving an optimal dose of thiopurines. Patients with ALL have to identify their phenotype and genotype before starting treatment with thiopurines. Due to the polymorphism of the enzyme the TPMT catalytic activity varies between patients, with most of variants showing an intermediate or
lower activity. Patients who are deficient in TPMT activity are of high risk developing life-threatening side effects if they receive standard doses of thiopurines (Wennerstrand P., 2012).

Today 44 different variants of the TPMT gene have been identified (TPMT allele nomenclature, 2015 [Online]; Lindqvist Appell M., et al., 2014).

A study performed on 7195 people in Sweden revealed that 89% were carrying the wild type TPMT, 10% had one nonfunctional allele and 0.5% were homozygotes with two nonfunctional alleles. The most common variants are TPMT*2 (A80P), TPMT*3A (A154T/Y240C) and TPMT*3C (Y240C) (Hindorg U., Lindqvist Appell M. 2012).

TPMT*6 and TPMT*8 have been classified as variants with low and intermediate enzyme activity in vivo studies. Surprisingly, in vitro studies showed that the enzymes activities are similar to the wild type or even higher in TPMT*6. Although TPMT*6 received a higher catalytic activity it turned out to be less stable than the wild type at physiological temperature (Wennerstrand P., 2012).

TPMT*6 (Y180F) has a mutation located in the hydrophobic core of the protein (Wu H., et al., 2007). This substitution probably disrupts the hydrogen bonding network since tyrosine is better than phenylalanine in making hydrogen bonds. Wennerstrand drew the conclusion that the low activity of TPMT*6 in vivo is caused by its decreased stability rather than loss of function due to the mutation.

The mutation in TPMT*8 is situated on the surface of the protein and shows similar stability to the wild type protein. Both TPMT*6 and TPMT*8 is slightly stabilized by the cofactor SAM, while ANS has a greater effect on the stability. Since the intermediate enzyme activity of TPMT*8 in vivo is not influenced by its stability it might depend on its interaction with SAM. However further experiments needs to be investigated for TPMT*8 (Wennerstrand P., 2012).

**Ligands**

**Thiopurines**

Thiopurines such as 6-mercaptopurine (6-MP, Figure 3), azathioprine (AZA) and 6-thioguanine (6-TG) are a group of antimetabolites structurally analogous to purines. The metabolism of these prodrugs results in the production of 6-thioguanine nucleotides (6-TGNs), metabolites which will be incorporated into DNA and RNA and induce apoptosis. This metabolic pathway involves series of different enzymes and an overview can be seen in Figure 4 (Wennerstrand P., 2012).

6-MP (and AZA which is converted to 6-MP) is either methylated by TPMT to the inactive metabolite 6-methylmercaptopurine or converted by the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) to thioinosine monophosphate (TIMP). If TIMP does not undergo conversion into 6-TGNs, it will be methylated again by TPMT into methyl-TIMP. Methyl-TIMP has an inhibitory effect on enzymes involved in de novo purine synthesis (DNPS) which further increases the cytotoxic effect by depletion of adenine and guanine nucleotides (Giverhaug T., Loennechen T., Aarbakke J. 1999).

The combination of 6-TGNs cytotoxicity and the inhibition of DNPS make thiopurines useful drugs for targeting rapidly growing tumor cells in ALL. Thiopurines also show immunosuppressive effects making it useful in treatment of inflammatory bowel disease and to prevent organ rejection after surgery.
Methotrexate

Methotrexate (MTX) is a folic acid analogue (Figure 5). This kind of antimetabolite shows immunosuppressant properties and an ability to inhibit de novo purine synthesis (DNPS). It is commonly used in treatment of various autoimmune diseases such as rheumatoid arthritis and in combination with 6-MP in treatments of ALL.

It is believed that MTX’s inhibition of the enzyme dihydrofolate reductase (DHFR) is its main mechanism of action. This inhibition prevents production of tetrahydrofolate from dihydrofolate which is required for synthesis of purines and the amino acids methionine and serine. Folates normally undergo a conversion into polyglutamyl forms of various lengths in the body. This conversion increase the cell's ability to store folates by increasing their water solubility and in addition seems to affect the binding affinity to different folate-dependent enzymes. The enzyme folylpolyglutamate synthase responsible for catalyzing the polyglutamation has been proven to have the same effect on MTX. This seems to be a contributing factor for MTX ability to inhibit DHFR. These formed methotrexatepolyglutamates (MTXPGs) are unable to cross the cell membrane and can therefore be stored in a much higher cytosolic concentration, which is necessary to be able to outcompete dihydrofolate properly (Chabner B. A. et al., 1985; Dervieux T. et al., 2002). However, if this polyglutamation affects MTX’s binding affinity in the same manner as folates remains to be investigated.
Fluorescence measurement showed that recombinant TPMTwt binds to MTX with a dissociation constant $K_d$ value of 40 $\mu$M (Wennerstrand P., 2012). Further experiments of different variants’ binding affinities are of high interest. Together with MTXPG studies more clues about the drug’s mechanism in the body can be revealed.

**Furosemide**

Furosemide (Figure 6) is a loop diuretic mainly used in treatment of edema and hypertension. It targets sodium potassium chloride co-transporters in the renal system. By inhibiting the uptake of ions in the ascending loop of Henle loop diuretics lowers the blood pressure and prevents buildup of water in the body. Furosemide has also been shown to have an effect on other organs apart from the kidney expressing for example vaso- and bronchodilative properties (Abbott L. M., Kovacic J., 2008). Studies also indicate that furosemide has an inhibitory effect on TPMT’s activity, which could lead to complications if co-administered alongside of 6-MP or AZA. This is mostly relevant for patients prescribed furosemide alongside of treatment with thiopurines against IBD or to prevent organ transplant rejections (Xin H. W. et al., 2005). The binding strength of furosemide to the selected TPMT variants can be investigated and compared to binding affinities for other drugs.

**8-anilinonaphtalene-1-sulfonic acid**

8-anilinonaphthalene-1-sulfonic acid (ANS, Figure 7) is commonly used as a fluorescent probe for water soluble proteins. It normally has a strong fluorescent emission when solved in a non-polar solution but is almost completely quenched by water solutions. ANS binds to hydrophobic sites on the surface of the protein. It interacts with its aniline-naphthalene group or by electrostatic interactions between positively charged residues on the protein and the negative sulfonate group on ANS, where the latter seem to be the major contributor. If this binding will protect ANS form the polar solvent and thus from the solvent’s quenching effect, an immense change in the fluorescence intensity will be observed (Matulis D. et al., 1999).

These properties make ANS an excellent probe which can be used for stability and unfolding studies. It can also be used to competitively determine ligand binding affinity for proteins by titration the ligand to a protein solution already saturated with the fluorescent probe. This is a useful method when the examined protein does not naturally contain any tryptophans close to the binding site (Angelakou A. et al., 1999).
Theoretical background for biophysical methods

Absorption spectroscopy

Light can be described as a beam of particles, photons, or as a wave function. A molecule is resting in its ground state $S_0$, which is the lowest energy level. When the molecule becomes exposed to a beam of light photons are absorbed and excites the molecule to a higher energy state. The frequency of the incident radiation and the energy required for transition must be equal according to Planck's equation (1) where $h$ stands for Planck's constant and $\nu$ for the light's frequency (Harris D., 2010).

$$E = h \nu$$ (1)

Since the excited state is not desirable the electrons will release its energy and return to the ground state via different relaxation transitions. It can be released through heat, collisions with other molecules or as emission of a photon. Some energy is always released as heat which is the reason that the wavelength of the emitted light is always longer than the absorbed one. This difference in wavelength is called Stokes shift. All biomolecules absorb energy at specific wavelengths depending on the particular electronic, vibrational and rotational energy levels of the molecule (Harris D., 2010). The major transitions in proteins involve aromatic side chains but also cofactors like chlorophyll, flavin and heme (Whitford D., 2005).

Absorption spectroscopy at 280 nm is a widely used method to determine protein concentrations. By measuring how much light that is absorbed by the sample a protein concentration can be calculated according to Beer-Lambert law (2), where $l$ is the light's path length through the sample, $c$ is the concentration and $\varepsilon$ the molar absorptivity specific for the wavelength (Harris D., 2010).

$$A = \varepsilon \cdot l \cdot c$$ (2)

Fluorescence spectroscopy

Fluorescence is a common method in the field of biochemistry. Lower concentrations are required compared to absorption spectroscopy making fluorescence a very sensitive method. Fluorescence occurs when excited molecules decay to the ground state via emission of a photon, where the spin multiplicity remains the same during the transition (Whitford D., 2005).

Collisions with other molecules shorten the lifetime of the excited state. This is known as quenching. The solvent is responsible for the majority of quenching through collisions with water molecules (Harris D., 2010).

Amino acids with aromatic side chains contribute to the majority of the fluorescence in proteins. Tryptophan is the strongest internal fluorophore, and due to its hydrophobicity it is often buried in the interior of the protein. Tryptophan fluorescence can be used for studying unfolding processes and monitoring interaction between protein and ligand. Both of these
methods can be seen as a change in intensity or as a wavelength shift. If a tryptophan is located in the vicinity of the binding site, a change in its local surrounding occurs upon binding. This change can be followed by titration of the ligand and a dissociation constant \( K_d \) can be calculated from the binding curve (Whitford D., 2005).

Since some proteins lack tryptophans an extrinsic fluorophore has to be used. The most common extrinsic fluorophore ANS binds hydrophobic cavities and fluoresces strongly in nonpolar environments. Competitive studies are widely used in pharmaceutical science where the protein is saturated with an extrinsic probe followed by titration of a drug. The drug outcompetes the fluorophore if it binds to the same binding site which can be seen as a decrease in fluorescence intensity (Angelakou A. et al., 1999).

TPMT consists of five tryptophans where at least two of them are located in vicinity of the active site (revealed by analyzing the TPMT structure using PyMol). This makes TPMT a suitable protein for tryptophan fluorescence measurements when studying ligand binding.

**Isothermal titration calorimetry**

Isothermal titration calorimetry (ITC) is a sensitive method used to study interactions between biomacromolecules such as protein to protein or ligand to protein binding. It has a capability to yield information about thermodynamic properties of the binding not available through other common methods used to determine binding affinities (Falconer R. J. et al., 2010).

The measurements are based on two cells encased in an adiabatic shield, one filled with the sample, the other containing a reference solution devoid of the studied molecules. These two cells are kept in thermal equilibrium. A ligand, commonly a small molecule, drug or metal-ion is loaded into a syringe attached to a very precise injection device. As the ligand is titrated into the sample the molecules interact and will either produce heat (exothermic) or require heat (endothermic). And thereby a temperature change in the sample cell will be induced. The calorimeter will register this change and compensate for it by adjusting the thermostats power supply in order to keep the temperature between the reference and the sample cell constant. The peaks from this signal, called the differential power \((DP)\), can be integrated with respect to time to calculate the enthalpy \((\Delta H)\) for the binding (Figure 8) (MicroCal user’s manual).

If the experiment is set up correctly, repeated injections will result in differential power peaks with decreasing intensity as the reaction reaches saturation. With this data an association constant \((K_a)\) and the number of binding sites \((n)\) can be calculated since the integrated peaks can be seen as a degree of
complex formation. Further thermodynamic information such as Gibbs free energy ($\Delta G$) and the binding entropy ($\Delta S$) can be obtained by combining equation (3) and (4).

$$\Delta G = -RT \ln K_a$$  \hspace{1cm} (3)
$$\Delta G = \Delta H - T\Delta S$$  \hspace{1cm} (4)

Further, if the experiment is repeated at several temperatures it is possible to calculate the heat capacity ($\Delta C_p$) of the binding, although this is less common since a single measurement is already time-consuming and require a substantial amount of protein (Saboury A., 2006; Falconer R. J. et al., 2010).

**Thiol-disulfide exchange**

Protein thiol groups, provided by the cysteine side chains are one of the most reactive functional group in proteins and a key in posttranslational modification. If two cysteine residues are brought near each other during folding they can form a disulfide bond. The disulfide bonds, known as cystine units contribute to the protein's tertiary and quaternary structure since they can form both intra- and intermolecular disulfide bonds. The formation of disulfide bonds are of great importance for the protein stability, structure and function (Winther J. R., Cholin T., 2014).

Detection of free thiol groups can be measured spectrophotometric through conjugation with 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB; also known as Ellman's reagent). This chromogenic compound consists of an oxidizing disulfide bond which can easily be reduced by free thiol groups. This exchange reaction between DTNB and free thiol groups produces equivalent amount of 5-thio-2-nitrobenzoic acid (TNB), this is illustrated in Figure 9. The absorbance of the TNB can be recorded spectrophotometric at 412 nm (Winther J. R., Cholin T., 2014).

![Figure 9: Reaction of DTNB with a available thiol-group. The formation of the free TNB molecule can be followed spectrophotometrically.](image)

TPMT consists of eight cysteines which generates an equal number of sulfhydryl groups in its reduced form. In its native form, only sulphydral groups on the surface of the protein are accessible for conjugation with DTNB. The rest of the cysteines which are either buried inside the structure or participates in disulfide bonds are unlikely to be reduced by Ellman's reagent (Lysaa R. A. et al., 2001).

Thiol-disulfide exchange of recombinant human TPMT has previously been investigated. Titration of the protein thiol groups with Ellman's reagent was performed by Roy A. Lysaa and coworkers. Their study revealed that at least two thiol groups are accessible for conjugation in its native active form. Some of the other cysteines are conjugated more slowly and seems to be located in the cofactor binding cleft where they are shielded by the cofactor SAM (Lysaa R. A. et al., 2001).
### Enzyme activity assay

Almost all chemical reactions in the body are assisted by biomolecules called enzymes. Most enzymes are proteins that catalyze reactions which normally occur to slow in the organism. This is done by stabilizing the transition state leading to an overall lowered initial energy required for the reaction to occur. A lowered starting energy allows the reaction to proceed at a considerable higher rate which is more suitable for the rapid metabolism observed in nature. Continuous evolutionary pressure has resulted in proteins containing sites in its surface designed to bind to certain substrates with high specificity. By orienting the substrates in an optimal way or by weakening or breaking important bonds with the use of a wide array of intermolecular forces the enzyme can create a very favorable environment for the reaction to occur. For many reactions the presence of the enzyme is not enough, as they are dependent on so called cofactors which assist catalysis. These cofactors are usually metal-ions or small organic molecules which can perform functions a protein itself cannot (Berg J. M., Tymoczko J. L., Stryer L., 2007). An example of this is SAM's donation of a methyl group in reactions catalyzed by TPMT as mentioned earlier.

By following the formation of the reaction product or the consumption of the substrate, usually done with radiochemical or spectrophotometric assays, it is possible to quantify the rate of the catalyzed reaction. This area of study is called enzyme kinetics. The Michaelis-Menten equation eq. (5) is commonly used as a mathematical description of enzyme kinetics. It is based on measuring the initial velocity of the reaction at different substrate concentrations producing a $V_{max}$ value. The maximal velocity is achieved when all of the enzymes exist in the enzyme-substrate complex, as well as Michaelis’ constant, $K_m$, which represents the substrate concentration when the reaction is carried out at half $V_{max}$ (Berg J. M., Tymoczko J. L., Stryer L., 2007).

$$v = \frac{d[p]}{dt} = \frac{V_{max}[S]}{K_m+[S]}$$  \hspace{1cm} (5)

If the reaction follows first-order enzyme kinetics, a Michaelis-Menten saturation curve Figure 10 can be constructed by plotting the velocity against substrate concentration. This results in a hyperbolic curve which often makes it hard to determine an exact value for $V_{max}$. The problem can be solved by constructing double reciprocal plot Figure 11 by inverting the values and using eq. (6) (Lineweaver H., Burk D., 1934)

$$\frac{1}{v} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$  \hspace{1cm} (6)

Radiochemical assays and HPLC techniques are common methods for monitoring the enzyme activity in vivo. Absorbance measurements at 320 nm using 6-MP as a substrate has been useful in in vitro studies (Wennerstrand P., 2012). Since these methods are quite time consuming they are now being replaced by faster methods like SAM Methyltransferase Assay (SAMfluoro™) (G-biosciences, 2012 [Online]). This expensive assay measures the amount of resorufin intensity which is a fluorescent product formed when TPMT converts SAM to SAH.
The amount of methylated substrate is equivalent to activity which in turn gives an estimation of the enzyme activity.

TPMT has previously been shown to have a catalytic effect on various thiophenol derivatives (Ames M. M. *et al.*, 1986). In 2002 L. M. Cannon *et al.* used the TPMT catalyzed methylation of the substrate 5-thio-2-nitrobenzoic acid (TNB) to investigate enantiomeric content in SAM supplements. This methylation induces a shift in the sample’s absorbance spectra which can be used to follow the consumption of the substrate during the reaction. TNB can be produced by reduction of the commonly available DTNB (Ellman’s reagent) with the reducing agent tris(2-carboxyethyl)phosphine hydrochloride (TCEP).

The possibility of using this reaction as an easy way to determine the catalytic activity of recombinant TPMT variants spectrophotometric was investigated. Although dependent on a more clinically irrelevant substrate, this method has the potential to be a quick assay to compare the quality of newly purified recombinant TPMT variants or confirm its existence in the properly folded native form. Even though the TNB method is slightly more inaccurate it could precede the use of more expensive and time consuming assays like radio ligands, 6-MP and SAMfluoro™ assays.

Figure 11: A double reciprocal plot, also known as a Lineweaver-Burk plot.
Materials

TPMTwt, TPMT*6 and TPMT*8 were all provided by the Department of Physics Chemistry and Biology at Linköpings University. They were purified by Patricia Wennerstrand according to the protocol described in her dissertation (Wennerstrand P., 2012).

SAM, TCEP, DTNB, ANS, MTX and furosemide were obtained from Sigma-Aldrich. MTXPG was kindly donated by Dr. Jacob Nersting, Rigshospitalet, Copenhagen, produced and purchased from Schircks Laboratories, Jona, Switzerland.

A potassium phosphate buffer pH 7.3 composed of 75 mM NaCl and 20 mM K₂PO₄ was used as reference, dialysis buffer and diluent in all experiments.
Experimental setup

Fluorescence spectroscopy

Fluorescence measurements were performed on a HoribaJobin Yvon FluoroMax-4 spectrofluorometer at 23°C. 1 ml of 1 µM TPMT protein was added to a Hellma Analytics Quartz SUPRASIL 10 mm cuvette. The protein was dissolved in a stock solution of 20 mM phosphate buffer.

Tryptophan fluorescence MTX saturation were measured on TPMTwt, TPMT*6 and TPMT*8. Aliquots of 1 µl MTX from a stock solution of 10 mM (dissolved in 0.1 M NaOH) were titrated directly to the cuvette resulting in MTX concentrations between 0 and 244 µM. TPMTwt was also titrated with 732 µM MTXPG. 14 aliquots of 10 µl each were added to the cuvette to a final ligand concentration ranging from 7.3 to 93 µM.

All measurements were performed with excitation wavelength at 295 nm, specific for tryptophan residues. The emission was recorded between 310-550 nm and the slits were set to 2.5 nm. Three accumulated spectra were measured on each titration step and used to produce a mean value. The experimental values were analyzed in GraphPad Prism. The change in fluorescence intensity recorded at 340 nm was normalized as a fraction of the initial intensity and then plotted against the increased concentration of MTX. A Kd value was then obtained by the fitting of a nonlinear specific binding curve.

Isothermal titration calorimetry

All experiments were performed on a Microcal VP-ITC MicroCalorimeter at 21°C. The software program VPViewer was used to select the correct parameters. The first injection per run added 3.0 µl with a duration of 3.6 seconds. The rest of the injections had a duration of 5.6 seconds and an injection volume of 7.0 µl. A total of 25 injections per run were carried out with a spacing of 300 seconds. The reference power was set to 8 µCal/sec.

Some preparations had to be done before the measurements. The proteins were dialyzed using a dialysis tube (Spectra/Por membrane MWCO: 12-14.000 flat width: 25 mm, diameter: 16 mm, vol/length: 2.0 ml/cm). 20 mM phosphate buffer was used for all dialysis and dilutions to obtain identical conditions. The instrument was cleaned with ultrapure water (Milli-Q) prior to the experiments and all solutions were degassed to avoid bubbles.

TPMT*6 (46.5 µM) and TPMT*8 (51.9 µM) was titrated with 1.0 mM ANS. A titration with furosemide was carried out for each variant. TPMT*6 (34.8 µM) was titrated with 0.75 mM furosemide. TPMT*8 (55.3 mM) was titrated with 1.0 mM furosemide. In all experiments, 1.8 ml protein was loaded to the sample cell and the syringe was filled with 300 µl ANS/furosemide.

All graphs and data were analyzed using the Origin 7 software and a Kd value could be calculated.
**Thiol-disulfide exchange**

The absorbance of TNB was measured on a *U2000 Spectrophotometer* from *HITACHI* at 412 nm using a *Hellma Analytics Quartz SUPRASIL* 10 mm cuvette. The TPMT protein had a final concentration of 3 µM in the cuvette, dissolved in 20 mM phosphate buffer. DTNB had a final concentration of 160 µM.

The first experiment was performed on TPMTwt. Thiol-disulfide exchange was investigated at its denatured, native and SAM-complexed form. SAM had a final concentration of 0.3 mM. The denaturant GuHCl had a final concentration of 6 M.

TPMTwt, TPMT*6 and TPMT*8 were then dialyzed to check the concentrations and to remove the reducing agent TCEP. Measurements of both the denatured and native form were investigated for all three variants. Measurements with addition of 0.5 mM TCEP in both the reference and the sample were performed on TPMT*8. The experiment was ran for both the dialyzed and non-dialyzed protein in its denatured, native and SAM-complexed form.

All spectra were corrected with a buffer blank composed of all components except the protein.

**Enzyme activity assay**

The absorbance of TNB was measured on a *U2000 Spectrophotometer* from *HITACHI* using a *Hellma Analytics Quartz SUPRASIL* 10 mm cuvette. The TPMT protein had a final concentration of 3 µM in the cuvette, dissolved in 20 mM phosphate buffer. SAM had a concentration of 0.15 mM.

1.0 mM TNB was prepared by adding 600 µl DTNB (10 mM) to a solution of TCEP. TCEP solution had been dissolved in 20 mM phosphate buffer to a final concentration of 10 mM.

A wavelength scan was performed between 300-500 nm with addition of 25 µM TNB (final concentration). Every scan had a duration of 2 minutes and was repeated 10 times.

The enzyme activity was measured at 410 nm for TPMTwt, TPMT*6 and TPMT*8. TPMT*6 had a concentration of 0.15 µM. Both TPMTwt and TPMT*8 had a four times higher concentration (0.6 µM) since they have lower activity than TPMT*6 *in vitro*. Experiments were run with 75, 50, 25 and 12.5 µl 1.0 mM TNB. Every measurement had a duration of 30 minutes.

The velocity of the enzyme catalyst was then plotted against TNB concentration using *Excel*.
Results and discussion

Fluorescence spectroscopy

The fluorescent measurements on MTX yielded an emission peak around 335-360 nm for all the variants. The intensity at 340 nm from each consecutive MTX addition was extracted and normalized as a fraction of the intensity change during the titration. The fractions were then plotted against the ligand concentration with a dilution effect taken into account. By fitting a nonlinear specific binding curve in the program GraphPad Prism a $K_d$ value for the binding of MTX to each of the TPMT variants was obtained, seen in Figure 12.

Figure 12: Data from each of the fluorescence measurements fitted with a specific binding curve in GraphPad.

The fluorescence measurements shows a small variance between the variants with a $K_d$ value of 43.5 µM for TPMTwt, 50.2 µM for TPMT*6 and 38.3 µM for TPMT*8. The obvious effect the addition of MTX has on the intensity seems to be a clear indication that MTX does indeed bind to TPMT. The affinity of MTX to TPMTwt has previously been investigated by Wennerstrand P. et al., 2012 where a $K_d$ value of 40 µM was received which corresponds very well with the obtained values above.

Both TPMT*6 and TPMT*8 shows a marginal deviation from the wild type, if this is due to inaccuracy in the measurements or to slightly different binding strengths is hard to determine,
especially with only a single measurement on each variant. However such a small difference in the affinity might not be all that relevant.

The same process was applied to the data from the MTXPG titration to TPMTwt, which revealed a $K_d$ value of 27.2 µM. This is reasonably lower when compared to that of the non polyglutamated MTX, indicating that this process does increase the binding affinity as suspected. However, the supply of MTXPG was very limited and depleted during the titration. As a consequence of this the protein was never completely saturated which means that the calculated $K_d$ value might be somewhat inaccurate. Unless solved together with high concentrations of NaOH, MTX is very poorly soluble in water solutions. This has led to problems during previous attempts to measure MTX binding affinity with ITC, a problem which could be circumvented by using the much more waters soluble MTXPGs.

Further investigations of MTXPGs binding to TPMT might be of high interest. A good place to start would be to measure the binding affinity to other TPMT variants since the small affinity differences between the variants might be amplified by the stronger binding of a polyglutamated ligand.

**Isothermal titration calorimetry**

Using the program *Origin 7* by *MicroCal* the data from the titrations was converted to a binding curve with a nonlinear fit, these graphs are shown in Figure 13 and Figure 14. This produced information about the stoichiometry (n-value) of the binding in addition of an association binding constant ($K_a$) which can be inverted to a $K_d$ value.

The ANS measurements resulted in a $K_d$ value of 0.86 µM for TPMT*6 and 0.37 µM for TPMT*8. This can be compared with measurements on TPMTwt performed by Borg A. and Göransson N. in 2013 which produced a $K_d$ value for ANS of 0.48 µM. The Furosemide measurements resulted in a $K_d$ value of 9.3 µM for TPMT*6 and 4.7 µM for TPMT*8. Again a comparison with binding for TPMTwt was provided by previous measurements done by Karlsson L. and Karlsson S. in 2014, with a $K_d$ value of 3.7 µM. The results from both ANS and furosemide show that the protein variants bind the ligands in a similar range as the wild type, with an indication that the binding to TPMT*6 is slightly weaker.

The number of binding sites (n-value) obtained for ANS were 0.57 and 0.48 for TPMT*6 and TPMT*8 respectively, and 0.21 and 0.40 for furosemide. TPMTwt measurements had a value of 0.87 for ANS and 0.69 for furosemide which is closer to the assumed number of one binding site.

It is likely that this deviation is due to the protein being partially denatured which would lead to a lower effective protein concentration. Modelling performed on the TPMT*6 and TPMT*8 data using a lower hypothetical protein concentration resulted in n-values closer to 1, but did not affect the $K_d$ value at all.

Worth noting is that during the measurement on furosemide to TPMT*6 both the protein and ligand solution were diluted with a factor of 0.75 since the volume of the protein did not meet the requirements for the measurement. This should not affect the resulted $K_d$ value but could contribute to its inaccuracy.
Figure 13: Results from ITC measurements with ANS. TPMTwt, TPMT*6 and TPMT*8 are shown from left to right.

Figure 14: Results from ITC measurements with furosemide. TPMTwt, TPMT*6 and TPMT*8 are shown from left to right.

The results from all the binding measurements are summarized in Table 1. A general trend can be observed which shows that TPMTwt and TPMT*8 binds all the ligands with a similar strength. For TPMT*6 the ITC measurements indicates a slightly weaker binding for both of the ligands, a trend which could perhaps be seen in the fluorescence data. TPMT*6 in known to have a much lower stability than TPMTwt, with a 5°C difference in thermal midpoint. This lowered stability could be reflected in the weaker binding observed (personal communication with Lars-Göran Mårtensson, Linköping University).

Table 1: Summary of binding dissociation constants obtained from fluorescence and ITC measurements.

<table>
<thead>
<tr>
<th>Variant</th>
<th>$K_d$ ANS ($\mu$M)</th>
<th>$K_d$ Furosemide ($\mu$M)</th>
<th>$K_d$ MTX ($\mu$M)</th>
<th>$K_d$ MTXPG ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPMTwt</td>
<td>0.48±0.1$^1$</td>
<td>3.7±0.6$^2$</td>
<td>43.5±3.2</td>
<td>27.2±3.2</td>
</tr>
<tr>
<td>TPMT*6</td>
<td>0.86±0.3</td>
<td>9.3±2.5</td>
<td>50.2±4.5</td>
<td>N.D.$^3$</td>
</tr>
<tr>
<td>TPMT*8</td>
<td>0.37±0.1</td>
<td>4.7±0.6</td>
<td>38.3±1.8</td>
<td>N.D.$^3$</td>
</tr>
</tbody>
</table>

$^1$Values obtained from Borg A. and Göransson N.
$^2$Values obtained from Karlsson L. and Karlsson S.
$^3$N.D. = Not determined.
Thiol-disulfide exchange

By calculating a value for the amount of formed TNB using the molar extinction coefficient 13600 M\(^{-1}\) cm\(^{-1}\) together with the measured absorbance and the known TPMT concentration, a value representing the amount of available thiol groups on the protein was received.

The first measurement was performed on TPMTwt in a denatured, native and SAM-complex form (Appendix). All investigated conditions resulted in much higher values than expected. Values higher than 8 should not be possible since TPMTwt only contain 8 cysteines in total. An assumption was made that the high absorbance was due to the formation of TNB throughout another process than DTNB’s reaction with the available thiol groups on the protein. Therefore all protein samples were dialyzed against phosphate buffer in order to remove TCEP in the original protein solution. The measurements performed after the dialysis resulted in the graphs seen in Figure 15.

![Graph: Results for dialyzed TPMT variants](image)

**Figure 15:** Spectroscopic measurements at 412 nm on each dialyzed variant.

The obtained intensities were very low, and the calculated thiol group equivalents were around 3 in the denatured and 1.8 in the native state for both TPMTwt and TPMT*6. TPMT*8 showed 4 available thiol groups in the denatured state and 1.3 in the native. In theory all of the 8 cysteines should be exposed when the protein is denatured, yet here it shows 3-4 available thiol groups. This indicates that even in the supposedly denatured state far from every cysteine is able to react with DTNB. This could perhaps be due to intermolecular interactions in form of disulfide bonds between the proteins. As such it seems that it is necessary to have some sort of reducing agent present during the experiment. Measurements with addition of TCEP were performed on dialyzed and non-dialyzed TPMT*8 in an attempt to negate the background contribution. Unfortunately it did not work as it became apparent that the TCEP concentration in the original protein solution was lower than initially thought. These results are illustrated in Appendix.

Even though it was not possible to produce results corresponding to the correct amount of thiol groups, it is still possible to use the data in Figure 15 to compare the internal relations between different TPMT variants. Both TPMTwt and TPMT*6 seems to be very similar, suggesting that this mutation does not lead to the formation of different amount of disulfide
bridges. If this was the case it could have been an explanation for the lower stability observed in TPMT*6 (Wennerstrand P., 2012). TPMT*8 differs compared to the other variants although it showed a similar stability to TPMTwt (Wennerstrand P., 2012). It is suspected that the deviation is due to inaccurate data since there was no time to repeat the measurements more than once. Another suggestion is that the high absorbance of denatured TPMT*8 is due to lack of a disulfide bond present in the other variants. However this would contradict the similarity in stability seen between TPMT*8 and TPMTwt.

A future prospect would be to use this methods for other variants such as the more common TPMT*3C containing the mutation Tyr240Cys. The introduction of a new cysteine residue makes this variant a much more likely candidate for the formation of new disulfide bonds.

**Enzyme activity assay**

The wavelength scan between 300-500 nm is shown in Figure 16. The decreasing absorbance at 410 nm confirmed that the methylation of TNB took place and the following measurements were performed as a time scan set with varied TNB concentrations. For each concentration and variant the decreasing absorbance was used to calculate the rate of the substrate consumption per second. These values were then plotted in a Michaelis-Menten saturation curve as seen in Figure 17. The data for TPMT*8 was omitted as the velocity decreased with increasing substrate concentration making it impossible to determine a $V_{\text{max}}$. It is known that SAH has an inhibitory effect on TPMT, which could be a reason for this trend. However, there is an uncertainty why this product inhibition is so prominent for only TPMT*8.

![Wavelength scan for TPMTwt](image)

Figure 16: A repeated wavelength scan performed on TPMTwt with 25µM TNB and an excess of SAM. The initial absorbance maximum seen at 410nm shifts to 350nm when TNB is methylated.
Figure 17: Data for each variants enzyme activity assay plotted in a Michaelis-Menten curve.

By using Lineweaver-Burk plots (data not shown) values for $V_{\text{max}}$ and $K_{m}$ were calculated for TPMTwt and TPMT*6 presented in Table 2.

Table 2: Calculated kinetic properties obtained from the enzyme activity measurements.

<table>
<thead>
<tr>
<th>Variant</th>
<th>$V_{\text{max}}$ (nM/s)</th>
<th>$K_{m}$ (µM)</th>
<th>$K_{cat}$ (s$^{-1}$)</th>
<th>$K_{cat}/K_{m}$ (s$^{-1}$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPMTwt$^1$</td>
<td>8.8$^2$</td>
<td>3.9</td>
<td>0.015</td>
<td>3.7 \cdot 10^3</td>
</tr>
<tr>
<td>TPMT*6$^3$</td>
<td>7.9</td>
<td>4.0</td>
<td>0.053</td>
<td>13.3 \cdot 10^3</td>
</tr>
</tbody>
</table>

$^1$ Total protein concentration of 0.6 µM  
$^2$ Data point for 75 µM TNB omitted.  
$^3$ Total protein concentration of 0.15 µM

These results are in agreement with previous published results (Wennerstrand P., 2012) which shows a higher catalytic activity for TPMT*6 in vitro.  
Although there might be some optimization needed, it is clear that this method can be used as an assay to determine TPMT’s enzymatic activity. Even though the quantitative results from this method might not be of very high interest as it represents a different kind of substrate, it shows great promise to be used qualitatively. The assay could be used to swiftly and cheaply determine if a recombinant produced enzyme has any activity directly after purification or after retrieving it from long time storage. By adapting the assay to usage with plate-readers one could quickly compare activity between new variants, at a much lower cost than SAMfluoro™. Perhaps it could even be adapted to clinical use on blood samples.
Conclusions

Our results confirm that each of the ligands binds all of the three examined variants. The binding strength is in a range between 0.3 to 50 µM with ANS showing the lowest K<sub>d</sub> value and MTX the highest. An overall trend can be observed, showing that TPMT*6 has a slightly higher dissociation constant for each ligand.

The experiments also showed that MTXPG<sub>7</sub> binds with a higher affinity to TPMT<sub>wt</sub> compared to MTX. Further studies on MTXPGs of different length are required in order to better understand how this conversion affects methotrexate’s interactions with folate dependent enzymes.

We have shown that it is possible to spectrophotometrically measure the enzymatic activity of TPMT with TNB as a substrate. If adapted to use with plate readers this method could in the future prove to be a fast and cheap alternative to current assays. Although the thiol-disulfide exchange experiment could be further optimized, it is possible to compare the amount of available thiol groups between different variants.
Acknowledgement

We wish to express our sincere thanks to Lars-Göran Mårtensson for giving us the opportunities to work with this project. We could not have imagined a better supervisor than you! Your calm and patience always rubs off on us and your support throughout this project has been invaluable.

In addition to providing the protein we would like to give Patricia Wennerstrand a special thanks, since a lot of our project has been based on her previous research.

Thanks to Madhanagopal Anandapadamanaban for his excellent tutelage, it took us through the ITC measurements with ease.

We would also like to thank Cecilia Andrésen for lending us her time and resources. Even in the busiest of times she always helps students with a smile on her face.
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Appendix

Figure 18: Results from thiol-disulfide exchange measurements on TPMTwt in its denatured, native and SAM-complexed form. The number of accessible cysteines was calculated to 18, 14 and 11 respectively.

Figure 19: Results from thiol-disulfide exchange measurements on dialyzed and non-dialyzed TPMT*8, with TCEP added in equal amounts to both the sample and the reference. The difference between both the denatured states and both the native states respectively were used to calculate a concentration for the original TCEP concentration in the non-dialyzed sample. This equaled a TCEP concentration of 0.25 mM instead of the assumed concentration of 0.5 mM.