STRUCTURE-FUNCTION STUDIES ON DUTPASES FOR KINETIC ANALYSIS
AND INHIBITOR TESTING

Characterisation of Homo sapiens and Mycobacterium tuberculosis dUTPases

Ph.D. thesis

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1. INTRODUCTION

1.1 The dUTPase catalytic reaction

2'-deoxyuridine 5'-triphosphate nucleotidohydrolases (EC 3.6.1.23), generally referred to as dUTPsases, catalyse the hydrolysis of dUTP into dUMP and pyrophosphate [1]:

\[
\text{N} \quad \text{O} \quad \text{O} \quad \text{OH} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{O} \quad \text{P} \quad \text{O} \\
\text{dUTP} \quad \text{N} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{O} \quad \text{OH} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{O} \quad \text{P} \quad \text{O} \\
\quad \text{+} \quad \text{H}_2\text{O} \quad \text{dUTPase (Mg}^{2+}) \quad \text{N} \quad \text{H} \quad \text{N} \quad \text{O} \quad \text{O} \quad \text{OH} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{O} \quad \text{P} \quad \text{O} \\
\quad \text{dUMP} \quad \text{+} \quad \text{P} \quad \text{O} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{O} \quad \text{P} \quad \text{O} \\
\text{Scheme 1. The catalytic reaction of dUTPase: Formation of dUMP and pyrophosphate by the hydrolysis of dUTP.}
\]

During the catalytic reaction, a water molecule within the enzyme active site carries out a nucleophilic attack on the α phosphorus atom hydrolysing the phosphoanhydride bond bridging the α and β phosphorus atoms. The cofactor of dUTPase is Mg\(^{2+}\), coordinating the substrate in the catalytically competent conformation [2, 3]. The equilibrium of the reaction is shifted far to the products (a high energy phosphoanhydride bond is cleaved), and given the presence of pyrophosphatase enzymes in the cells, may be pulled in vivo even further towards that direction by the consumption of pyrophosphate [4].

1.2 Structural architecture

The importance of dUTPase is highlighted by the fact that most organisms encode a dUTPase sequence in their genomes [5]. The 3D crystal structures of several of these dUTPases have been determined and are available in the PDB (some PDB IDs: *Escherichia coli* 1EU5 [6], *Feline immunodeficiency virus* 1F7D [7], *Homo sapiens* 1Q5U [8], *Mycobacterium tuberculosis* 1MQ7 [9]). These structures reveal that, interestingly, most dUTPases share the same overall fold, quaternary, tertiary and even secondary structural elements; this structure is evolutionarily well conserved [10, 11]. DUTPases are homotrimers with three identical active sites located at the subunit interfaces [10]. The monomers usually
possess 150 residues containing the following five highly conserved motifs: Motif I: Ala-Gly- (Pho)-Asp-Leu; Motif II: (Pro/Gly)-(Arg/Lys)-Ser; Motif III: Gly-(Pho)_2-Asp-(Nnn)_2-Tyr- (Nnn)-Gly; Motif IV: Arg-(Pho)-Ala-Gln; and Motif V: Arg-Gly-(Nnn)_2-Gly-Phe-Gly- (Ser/His)-(Thr/Ser)-Gly (amino acids in three-letter codes: Pho, hydrophobic residue; Nnn, any residue) [12] (Figure 1.). A minority of dUTPases is monomeric, but they also contain the 5 motifs [13].

![Alignment of dUTPases from different species.](image)

Each dUTPase subunit folds into antiparallel β sheets with the C-terminal β strand extended outside the subunit domain boundary (Figure 2. A). The domain fold is a distorted eight-stranded β sheet consisting primarily of a six-stranded antiparallel β jelly roll. Each active site is built up by the swapped motifs of the three monomers [15]. Motif 1, 2 and 4 are coming from one monomer (jelly-roll domain), Motif 3 is from the second monomer, forming a β-turn which is interlocked into the barrel. The C-terminus containing Motif 5 flanks over the active site pocket. Interactions between the residues of Motif 3 (mostly hydrogen bonds) with the uracil deoxyribose nucleoside segment of the substrate provide the exclusive recognition of the deoxyribose ring as well as the specific discrimination between uracil and the other two bases of the pyrimidine nucleotides. Thus, hydrogen bonds and steric interactions within the β-hairpin (involving Motif 3) form the specific recognition pocket for...
the uracil deoxyribose nucleoside discriminating against all the other nucleotides [8] (Figure 2, B and C).

A significant difference between prokaryotic and eukaryotic dUTPases is the polarity of the central channel, i.e. the main subunit interface [14]. In prokaryotic dUTPases, residues within this central channel are hydrophobic, that is, there are mainly van der Waals interactions keeping the subunits together here, making the channel quite tight and practically inaccessible to water molecules [10]. Eukaryotic dUTPases, however, tend to have polar central channels, where the main interactions are dipole-dipole interactions either between the subunit residues or, very importantly, between the amino acid side chains and solvent water molecules [14].

Figure 2. Crystal structure of FIV dUTPase in complex with dUDP (PDB ID: 1F7R). A. The overall fold of the dUTPase homotrimer. The five conserved motifs are in magenta. B. Close-up of the active site. The five conserved motifs are in magenta. Motif 1, 2 and 4 are from monomer A, motif 3 is from monomer B, and motif 5 is from monomer C [7]. C. Interaction mapping in enzyme-substrate complexes. Interactions are shown only for the phosphate chain moiety of the ligand [3].
1.3 The significance of dUTPase: thymidylate metabolism, thymine-less cell death

The dUTPase reaction has a dual role within the cell [16]: On one hand, it decreases the cellular dUTP level, on the other hand, it provides the basis of dTTP biosynthesis by producing dUMP, that is subsequently methylated by thymidylate synthase to give dTMP, which is phosphorylated up to dTTP in two steps (Figure 3.) [17]. Thus, it takes part in the regulation of the metabolism of two nucleotides (dUTP and dTTP) in one reaction, preferentially keeping a low dUTP/dTTP ratio [5] (which is 0.05-0.07 in human cells [18]).

![Figure 3. Simplified scheme of de novo dTTP biosynthesis.](image)

The two key enzymes regulating the dTTP/dUTP ratio are dUTPase and thymidylate synthase. dCMP deaminase has not been identified in enterobacteria, though it is present in most eukaryotic organisms, while dCTP deaminase has only been identified in prokaryotes [4]. Enzyme function deficiency results in an elevated dUTP/dTTP ratio, and a highly uracil-substituted DNA, due to the low substrate specificity of DNA polymerases [19, 20], and the relatively increased level of deoxyuridine triphosphate. Since uracil also appears in the DNA by the spontaneous oxidative deamination of cytosine [21], and this base replacement introduces a point mutation into the DNA, base excision repair enzymes remove uracil generating an abasic site and a single strand nick. Although the replacement of thymine
by uracil in the DNA, which becomes excessive in dUTPase deficiency, would not be mutagenic by itself, base excision repair enzymes usually cannot distinguish between uracil bases by their origin, and consequently remove all uracils from the DNA. High dUTP concentration results in the repeated misincorporation of uracil instead of thymine, and thus a high number of nicks in the DNA strands as a result of the hyperactivated base excision repair enzymes, leading to DNA fragmentation and a subsequent cell death. This process is known as thymine-less cell death [22, 23].

1.4 Therapeutic potential

Enzymes of de novo thymidylate biosynthesis are often targeted by drugs in anticancer and antiviral chemotherapy, e.g. fluorouracil against thymidylate synthase or methotrexate against dihydrofolate reductase [24]. These drugs induce thymine-less cell death by perturbing thymidylate metabolism, since cells with actively ongoing DNA synthesis, e.g. tumour and virus-infected cells, are much more sensitive against it [25]. In E. coli and in S. cerevisiae, lack of dUTPase was proved to be lethal [26, 27]. The importance of thymine-less cell death is underlined by the fact that this apoptotic pathway was found to be independent from p53-signaling in several human tumour cell lines [28, 29]. In human cancer cells, dUTPase has been proposed to be an important survival factor desensitising tumours against drugs perturbing thymidylate metabolism [30, 31]. Targeting dUTPase has therefore been suggested as a promising approach in combination with drugs already in clinical use against thymidylate synthase and dihydrofolate reductase [32].

The therapeutic potential of inducing thymine-less cell death could be exploited not only in human cancer cells, but also in another species exerting an alarmingly rising threat to human population, namely Mycobacterium tuberculosis.

1.5 Tuberculosis

Tuberculosis (TB) is one of the most abundant and deadliest infectious diseases with one third of the human population already infected [33]. After some successful chemotherapeutic agents had been developed by the end of the 1960s, this disease was
thought to be overcome, and there was no great effort in the design of novel, more efficient drugs. Soon after the introduction of the first anti-tuberculosis drug, streptomycin (1943), drug-resistant strains appeared [34]. The subsequent introduction of para-aminosalicylic acid [35] and isoniazid [36] helped the situation, and the appearance of rifampicin in 1965 seemed to have solved the problem completely [37]. The uncontrolled treatment and unsatisfying compliance to the therapy, however, has led to the rise of multi-drug resistant tuberculosis incidence (MDR-TB; defined as resistance to isoniazid and rifampicin and possibly other drugs) [38]. Moreover, the poor compliance to the therapy using these drugs combined with the second-line antituberculotics (e.g. pyrazinamide and ethambutol) resulted in the appearance of extensively drug-resistant strains (XDR-TB; resistant to at least three of the available antituberculotics including rifampicin and isoniazid), and the number of such cases is still increasing alarmingly [39]. The emergence of such cell lines is a real challenge and urges the development of novel drugs [40].

1.6 Characteristics of human and M. tub. dUTPases

In order to be able to design effective inhibitors, however, it is essential to reveal the detailed enzymatic mechanism, and to learn about the structural and functional characteristics, and thus find the most powerful approach of enzyme inhibition.

1.6.1 Human dUTPase

The human enzyme, like most dUTPases, is a homotrimer with five, highly conserved motifs and a structure described above, as revealed by its first crystal structure [8]. Two isoforms of human dUTPase were identified in human cells, which are encoded by the same gene with isoform-specific transcripts arising through the use of alternative 5’ exons [41]. The two isoforms are largely identical, differing only in a short region of their amino-terminal sequences, which contain distinct localisation signals for both isoforms. Thus, the two isoforms of human dUTPase have distinct cellular localisations; the shorter one is localised to the nucleus (hDUT-N), and was found to be approximately 30 times more abundant in dividing cells than the longer isoform (hDUT-M), that is localised to the mitochondrion. It
was shown *in vitro*, that a precursor protein of hDUT-M is processed and imported into mitochondria [42]. A posttranslational modification exclusive for hDUT-N was also found: Ser11 is phosphorylated [43]. The expression pattern was also found to be different: while hDUT-M was found to be expressed constitutively, a cell cycle dependent expression level was observed for hDUT-N. The expression of the nuclear isoform was found to be upregulated during nuclear DNA replication, while it was hardly detectable in resting cells [41]. Despite the structural and regulatory differences, both forms of dUTPase exhibited identical $K_M$ values of 2.5 $\mu$M for dUTP (as determined from samples purified from cellular extracts) [42].

Apart from this series of thorough studies addressing cellular level and regulation of physiological isoforms, not much enzymological information had been available, particularly not about the catalytic mechanism and active site structure. A typical phenomenon for dUTPase crystal structures, i.e. the invisibility of the C-terminal arm suggested to be crucial for catalysis [44], applied to the human enzyme, as well. At the same time, this segment was found to be quite flexible in several dUTPases and its ordering (closing upon the active site pocket) was thought to be induced by substrate binding [8, 45-48]. It was shown that in the prokaryotic dUTPase, this segment readily opens up after the catalytic cleavage [49]. However, in eukaryotic *D. mel.* dUTPases, arm closure/opening in one active site was reported to be dependent on the ligand-binding state of the other two active sites [45]. Furthermore, the catalytic mechanism of the prokaryotic enzyme was proven to follow a regular Michaelis-Menten scheme, while there had been reports of some kind of allosterism in the case of *D. mel.* dUTPases [45, 46] (Figure 4.).

Based on these differences, the possibility of allosterism/cooperativity for eukaryotic dUTPases emerge in contrast to the prokaryotic enzymes.
Figure 4. The catalytic cycle of pro- and eukaryotic dUTPases [14]. Left panel illustrates the catalytic cycle of E. coli dUTPase. After substrate binding and the subsequent hydrolysis step, the C-terminal arm readily opens up to release the products. In the right panel, the schematic catalytic cycle illustrates that even after the hydrolysis step, there is an altered conformation as compared to the prokaryotic enzyme. The other significant difference between pro- and eukaryotic dUTPases is the polarity of the central channel, which is denoted with grey for the former one indicating its apolar character, while it is denoted with orange in the latter one to indicate its polar character.
1.6.2 *Mycobacterium tuberculosis* dUTPase

The first report on *M. tub.* dUTPase crystal structure gave a detailed description of the interactions of the active site, which were found to be quite similar to the ones in other dUTPases [3, 9]. However, due to flexibility of the C-terminal region, the last ten residues could not be localized. As discussed above, this is a usual phenomenon in the determined dUTPase crystal structures; few structures could visualise this segment, which is part of motif 5 and, in *M. tub.* dUTPase, it interestingly contains a His residue at the site of an otherwise strictly conserved Phe. The sequence of *M. tub.* dUTPase also reveals a 4-residue insertion as compared to the human sequence just before Motif 5, and another species-specific feature is the C-terminal tetrapeptide. This study also proposed a mechanism for nucleophilic attack by an activated water molecule in agreement with in-depth structural and kinetic studies performed with wild-type and active-site-mutant *E. coli* dUTPase complexes [3].

The significance of dUTPase in *Mycobacterium tuberculosis* is emphasised by its key role in the dTTP biosynthesis. Neither dCMP deaminase nor dT kinase have been identified in the *M. tub.* genome, so there seems to be no alternative pathway for dUMP formation, but through the dCTP → dUTP → dUMP reaction pathway (cf. Figure 3.). The crucial availability of dUMP for dTTP biosynthesis in this bacterium is well illustrated by the fact, that a bifunctional dCTP deaminase–dUTPase has recently been identified in *Mycobacterium tuberculosis*, although it exerted a very low $k_{cat}$ for dUTP ($k_{cat} = 0.33 \pm 0.02 \text{ s}^{-1}$) [50]. Thus, the dUTPase function provides the sole *de novo* source of dTMP for DNA biosynthesis making dUTPase a potential target. In order to become a real target, however, the presence of species-specific features is crucial for specific targeting and inhibition.
2. AIMS

The present study aimed to achieve the followings:

1) Comparing the structural properties of human dUTPase to \textit{E. coli} dUTPase to determine whether the differences between \textit{E. coli} and \textit{D. mel.} dUTPases can be extended to prokaryotic vs eukaryotic dUTPases by:
   - Crystallising the protein to analyse the C-terminus and its interactions.
   - Characterising the human dUTPase in solution to compare the results to those gained on the \textit{E. coli} dUTPase.
   - Analysing the 3D crystal structure with a special focus on the C-terminal arm to determine its role in catalysis.

2) Revealing the detailed kinetic mechanism of human dUTPase to decide whether there is any kind of allosterism/cooperativity as hypothesised for the \textit{D. mel.} enzyme by both
   - equilibrium measurements (binding studies),
   - transient kinetic measurements.

3) Identifying species-specific segments in the \textit{M. tub.} dUTPase structure, and developing activity assays for large scale inhibitor testing by:
   - Analysing the 3D crystal structure to identify the species-specific segments that might be targeted.
   - Creating a sensitive label that reports about active site conformational changes.
   - Creating an efficient, sensitive and reliable activity assay potent even for large-scale, high-throughput screening that enables the fast screening of potential inhibitors.
3. MATERIALS & METHODS

3.1 Materials

Electrophoresis materials were obtained from BioRad, other chromatography resins and columns were purchased from Amersham Biosciences, UK. Phenol Red indicator and ammonium bicarbonate were obtained from Merck, Germany. Nucleotides, and other chemicals of analytical grade purity were from Sigma, US. α,β-imido-dUTP and 2-amino-6-mercapto-7-methylpurine ribonucleoside were purchased from Jena Bioscience, Germany. NiNTA resin was purchased from Novagen, Germany. Restriction enzymes and other molecular biology materials were purchased from Stratagene, US, unless stated otherwise.

3.2 Cloning and mutagenesis

The nuclear isoform of human dUTPase (hDUT):
The cDNA encoding the nuclear isoform of human dUTPase (a kind gift from Dr. Robert Ladner) was cloned into a pET3a plasmid by conventional PCR amplification by Dr. Júlia Kovári in our laboratory. However, the resulting DUT-N-pET-3a plasmid was recloned to obtain the N-terminally His-tagged dUTPase (His-hDUT). This new plasmid was the template when the Phe158Trp mutation was introduced by Quikchange using the forward primer: 5’-GGGGTTCAGGAGGTTGGGGTTCCACTGGAAAG-3’ and the reverse primer: 5’-CTTTCCAGTGGAACCCCAACCTCCTGAACCCC-3’. This plasmid was then further mutated – replacing Asp102 to Asn – to create the inactive hDUT158W;102N double mutant by Quikchange using the forward primer: 5’-GGAGCTGGTGTCATAATGAAGATTATAGAGGAATGTTGG-3’ and the reverse primer: 5’-CCACATTTCTCTATATACTTCATTTTATGACACCAGCTCC-3’.

The His-tagged Mycobacterium tuberculosis dUTPase (His-mtDUT) and its single His145 Trp mutant (His-mtDUT145W) was cloned by Enikő Takács in our laboratory as follows: The gene encoding for the dUTPase was amplified by PCR from the genomic DNA of Mycobacterium tuberculosis H37Rv strain using the forward primer 5’-
GGGAATTCCATATGTCGACCACTCTGGCGATCGTCCGC-3’ and the reverse primer 5’-CGCGGATCCTCACAAACTCGCATGTCCGCCGGAGGA-3’, the former one also containing an NdeI restriction site (CATAT), while the latter one containing a BamHI restriction site (GGATCC), and both containing digestion helping sequences (GGGAATTCC and CGC for the forward and the reverse primers, respectively). The amplified dUTPase gene was digested with NdeI/BamHI. An additional fragment was cut out from the plasmid pET-3a (Novagen) with BamHI/PstI. The digested dUTPase gene sequence then, together with this additional fragment, was ligated into a plasmid pET-3a predigested with NdeI/PstI. The resulting DUT-pET-3a plasmid was digested with NdeI/PstI and was ligated into a pET-19b plasmid predigested with NdeI/PstI.

Cloning was checked by DNA sequencing (MWG Biotech, Germany) on both strands for all the plasmids.

### 3.3 Expression and Purification

The plasmids were transformed into an *E. coli* BL21(DE3) (pLysS) strain for protein expression. The non-tagged enzyme was expressed and purified according to [46]. The other enzymes were expressed as described shortly: The final supernatant after cell extraction was loaded on a NiNTA column and purified according to the Novagen protocol. Stepwise washing was applied; after the salty washing steps, a 50 mM TrisHCl pH 7.5 buffer containing 30 mM NaCl and 50 mM imidazole washing step was applied to elute aspecific binding proteins. dUTPase was eluted with 0.5 M imidazole pH 7.5, and was subsequently dialysed into the appropriate buffer.

### 3.4 Analytical gel filtration

Depending on enzyme purity (usually in the case of the non-tagged species and for crystallography), gel filtration was also employed on a Superdex 200 HR column calibrated with bovine serum albumin, ovalbumin, chymotrypsinogen and RNase (molecular masses: 67, 43, 25 and 13.7 kDa, respectively).
3.5 **SDS-PAGE, concentration determination**

SDS-PAGE was performed according to [51] using 12% polyacrylamide minigels in the Protean III apparatus (BioRad). Protein bands were visualized by colloidal Coomassie Brilliant Blue staining (Biosafe Coomassie stain, Bio-Rad) and quantitative analysis was done by densitometry on a GelDoc densitometer (BioRad).

Protein concentration was measured either by Bradford’s assay [52] (using the protein determination kit from Sigma, and bovine serum albumin as standard), or spectrophotometrically using a JASCO-V550 spectrophotometer and a 10 mm pathlength cuvette thermostatted at 25°C. Molar extinction coefficients were calculated from the amino acid composition for the monomers (using the PROTPARAM program at [http://au.expasy.org](http://au.expasy.org)). Values are listed in Table 1. below. Throughout the whole thesis, concentrations always refer to monomer concentrations.

Table 1. Summary of molar extinction coefficients of the different recombinant protein constructs.

<table>
<thead>
<tr>
<th></th>
<th>hDUT</th>
<th>His-hDUT</th>
<th>His-hDUT^{158W}</th>
<th>His-hDUT^{158W,102N}</th>
<th>His-mtDUT</th>
<th>His-mtDUT^{145W}</th>
</tr>
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<tr>
<td>$\varepsilon_{280 \text{ nm}} / M^{-1} \text{ cm}^{-1}$</td>
<td>9080</td>
<td>10430</td>
<td>15930</td>
<td>15930</td>
<td>2980</td>
<td>8480</td>
</tr>
</tbody>
</table>

3.6 **Activity assays**

i) Phenol red indicator assay was performed as described in [53], using a JASCO-V550 spectrophotometer and a 10 mm pathlength cuvette thermostatted at 25°C.

ii) NADH coupled assay: The absorbance change of the $\text{NADH} \rightarrow \text{NAD}^{+}$ transition was followed at 340 nm in a 10 mm pathlength cuvette thermostatted at 25°C using a JASCO-V550 spectrophotometer. The experimental conditions were: $\text{lactate dehydrogenase} = 68 \text{ U/mL}$, $\text{pyruvate kinase} = 143 \text{ U/mL}$, $\text{[NADH]} = 100 \text{ mM}$, $\text{[phosphoenol pyruvate]} = 100 \text{ mM}$, $\text{[dUMP]} = 90 \text{ mM}$, $\text{[ADP]} = 100 \text{ mM}$ in 100 mM HEPES, pH 7.5, 100 mM KCl, 5 mM MgCl$_2$ buffer. Lactate dehydrogenase, pyruvate kinase, NADH and phosphoenol pyruvate
were added to the buffer, mixed and allowed to equilibrate at 25°C for a few minutes. Then measurement was started, and the reaction was initiated by the quick (less than 3 sec) addition of dUMP (detection interrupted). Since no signal was observed for more than 7 minutes, ADP was added as a control, which proved that the reaction conditions were appropriately set up. Since dUMP did not prove to be a substrate of pyruvate kinase, the set up was not optimised.

ii) Pyrophosphate assay: The EnzCheck Pyrophosphate Assay Kit was the initial basis of the assay (based on the method of Webb [54]). This kit, however, is only for end-point titration, and thus it could not be used for continuous detection. The concentrations had to be optimised so that the hydrolysis of dUTP would be the rate-limiting step. To follow the reaction continuously, inorganic pyrophosphatase, purine nucleoside phosphorylase, and 2-amino-6-mercaptopo-7-methylpurine ribonucleoside in particular, were required in much higher amounts than provided by the kit. Therefore, the enzymes inorganic pyrophosphatase and purine nucleoside phosphorylase were purchased from Sigma and 2-amino-6-mercaptopo-7-methylpurine ribonucleoside was purchased as a custom synthesis product from Jena Bioscience. The optimised conditions in the cuvette were: [dUTPase] = 100-180 nM, [inorganic pyrophosphatase] = 5 U/mL, [purine nucleoside phosphorylase] = 5 U/mL, [2-amino-6-mercaptopo-7-methylpurine ribonucleoside] = 100 µM, [dUTP] = 5 µM in 50 mM TrisHCl, pH 7.5, 1 mM MgCl₂ buffer using a JASCO-V550 spectrophotometer and 10 mm pathlength 25°C thermostatted cuvettes. 2-amino-6-mercaptopo-7-methylpurine ribonucleoside, purine nucleoside phosphorylase, inorganic pyrophosphatase and dUTP were added to the buffer, mixed and allowed to equilibrate at 25°C for a few minutes. Then measurement was started, and the reaction was initiated by the quick (less than 3 sec) addition of dUTPase (detection interrupted). The $k_{cat}$ values were calculated using the equation: $k_{cat} = \frac{v_{init}*[dUTP]}{\Delta A_{total}*[dUTPase]}$ for both this and the phenol red assay. Initial velocity was determined from the first 10% of the progress curve (cf. [55]).

3.7 Limited trypsinolysis

Limited trypsinolysis maps the structural hierarchy of the trimeric protein and provides information on possible flexible segments.

Limited trypptic digestion was carried out at 25°C, using 28 µM dUTPase and 1:250 (w/w) trypsin:dUTPase ratio in 10 mM sodium-phosphate pH 7.5 buffer containing 150 mM NaCl, 5
mM MgCl$_2$ and saturating concentrations of ligands, if present. Aliquots were taken (stopping the digestion by the addition of 1 mM phenyl-methane-sulfonyl-fluoride) at different time points for activity measurements (initial velocity determinations) and for SDS-PAGE and the subsequent MS analysis of the separated fragments.

### 3.8 Differential Scanning Microcalorimetry

Differential scanning calorimetry provides direct quantitative thermodynamic data on protein stability, and also indicates the oligomeric status of the protein during thermal unfolding. Differential scanning microcalorimetry was performed using a Microcal VP-DSC between 20 and 80°C at a scan rate of 1°C /min on a 12 μM dUTPase sample in 20 mM TES/HCl pH 7.5, 0.3 M NaCl, 1 mM dithiothreitol, 5 mM MgCl$_2$ buffer. Solutions were degassed by stirring under vacuum for 15 minutes at room temperature immediately prior to measurement. The solutions in the measuring cells were kept under pressure to prevent any bubble formation during heating. The baseline was determined in an identical experiment with buffer in both cells and was subtracted. The melting temperature ($T_M$) was defined and taken to be the maximum of the excessive heat capacity function. Calorimetric enthalpies were calculated as equal to the area under the heat absorption curves; molar calorimetric enthalpies were calculated by taking into account the molar concentration of the protein sample. The measured heat capacity functions were fitted with theoretical models assuming simple two-step transitions (molecule population consisting of either folded or unfolded species with no experimentally observable intermediates) and reversibility of the transition. Fitted model curves were used to calculate van’t Hoff enthalpies of the transitions. Data evaluation were performed using the built-in Origin software (MicroCal).

### 3.9 Isothermal Titration Calorimetry

Isothermal titration calorimetry is the most direct method for the determination of heats of binding ($\Delta H_{\text{binding}}$) and binding constants ($K_A$) upon macromolecular ligand binding. By performing isothermal titration calorimetry experiments, it is sometimes possible to determine
whether binding sites in oligomeric proteins show independent binding behaviour or whether possible cooperative interactions are reflected in the binding characteristics.

Isothermal titration calorimetry experiments were carried out under different conditions (see below) on a Microcal VP-ITC instrument. The titration was performed with injections of 3-16 µl from the syringe (containing the stock solution of α,β-imido-dUTP) to the cell (filled with dUTPase solution). Control experiments were performed with buffer in the cell in order to take into account the heats of mixing and dilution. Integration of heat signals and fitting of binding isotherm were done using the built-in Origin software (MicroCal). The built-in fitting models of the software use the following independent parameters: ‘One Set of Sites’ model: \( n, K, ΔH \); ‘Two Sets of Sites’ model: \( n_1, K_1, ΔH_1; n_2, K_2, ΔH_2 \); ‘Sequential Binding Sites’ model: \( K_1, ΔH_1; K_2, ΔH_2; K_3, ΔH_3 \), where \( n \) is the stoichiometry, \( K \) is the (association) equilibrium constant and \( ΔH \) is the molar enthalpy.

Conditions:

i) \( T = 28°C; 1.7 \text{ mM } α,β-\text{imido-dUTP stock solution;} 270 \text{ µM dUTPase solution;} \text{ buffer: 25 mM sodium phosphate pH 8, 1 mM MgCl}_2. \)

ii) \( T = 28°C; 2.2 \text{ mM } α,β-\text{imido-dUTP stock solution;} 270 \text{ µM dUTPase solution;} \text{ buffer: 20 mM HEPES pH 7.5, 150 NaCl, 1 mM MgCl}_2. \)

iii) \( T = 20°C; 2.2 \text{ mM } α,β-\text{imido-dUTP stock solution;} 270 \text{ µM dUTPase solution;} \text{ buffer: 20 mM HEPES pH 7.5, 150 NaCl, 1 mM MgCl}_2. \)

### 3.10 Fluorimetry

Fluorescence is a dominant and widely used methodology in life sciences, and many other fields of science. Fluorescence detection is highly sensitive and gives way to both equilibrium and transient kinetic measurements.

Emission spectra and fluorescence intensities were recorded on a Jobin Yvon Spex Fluoromax-3 spectrofluorimeter in a 10 mm pathlength cuvette thermostatted at 20°C. Enzyme concentrations were 1 µM, the buffer was either 20 mM TrisHCl, pH 7.5, 1 mM MgCl\(_2\) and 150 mM NaCl, or 20 mM sodium phosphate, pH 7.5, 1 mM MgCl\(_2\) and 150 mM NaCl. Excitation wavelength was 295 nm (slit = 2 nm), emission was scanned between 300 and 450 nm (slit = 5 nm). Three scans of every spectrum were averaged. Titration was carried out by the stepwise addition of 1–5 µl aliquots from concentrated nucleotide solutions, and the
emission intensity at 355 nm was followed on a time course for the human constructs, while the same was done at 354 nm for His-mtDUT$^{145W}$. Because large concentrations of nucleotides were used, care was taken to correct for any additional fluorescence or inner filter effect imposed on the measured intensities by the nucleotide stock solutions.

### 3.11 Transient kinetics

The stopped-flow is a rapid mixing transient kinetic technique in which the progress of a reaction can be followed optically on a millisecond timescale. The reagents are in different syringes that are pushed by a computer-driven motor simultaneously. The contents of the syringes are mixed in a cuvette where the biochemical reaction can be followed theoretically from the $t = 0$ time. In practice, each apparatus has a dead time during which data is not recorded and therefore is lost. The dead time (around 1 ms) gives a practical limit to the reaction rates that can be measured by stopped-flow.

Measurements were done using a SF-2004 (KinTek Corp., Austin, TX) stopped-flow apparatus. The change in absorbance at 546 nm selected with a monochromator (slit: 4 nm) detected by a photodiode was followed in 25 µM HEPES pH 7.5 buffer containing 150 mM KCl, 1 mM MgCl$_2$ and 40 µM phenol red at 20°C. Depending on reproducibility, data of three to five scans of each curve were averaged.

Tryptophan fluorescence was excited at 297 nm and emission was selected with a band-pass filter having a peak in transmittance at 340 nm at 20°C. Depending on reproducibility, data of five to seven scans of each curve were averaged.

Time courses were analyzed using the curve fitting software provided with the stopped-flow apparatus or by Origin 7.5 (OriginLab Corp., Northampton, MA).
4. RESULTS & DISCUSSIONS

4.1 Expression and purification (ref. 1 & 3 in the Publication List)

The clone hDUT in a pET-3a vector was overexpressed in *E. coli* BL21(DE3) strains, which resulted in a total amount of around 20 mg protein from cells grown in 1 L Luria-Bertani broth. This recombinant human dUTPase was used in the experiments at a purity higher than 90%. The His-tagged version of the same species was also constructed. The purification of this species was much shorter and more convenient, and additionally, it also increased purity above 95%.

All the His-tagged species were expressed in *E. coli* BL21(DE3) (pLysS) strains yielding 50-80 mg of proteins from 1 L Luria-Bertani broth depending on the species. Thus, a high-yield overexpression system for the nuclear isoform of human dUTPase, its His-tagged recombinant version, two His-hDUT mutants, the His-tagged mtDUT, and its mutant was successfully created. Figure 5 illustrates the different purification results of the non-tagged and the His-tagged species. His-hDUT was also found to be less sensitive to degradation as compared to its non-tagged form, probably due to the 6xHis aminoacids stabilising the otherwise quite flexible and easily degradable N-terminus.
Figure 5. The different expression and purification of the non-tagged and the His-tagged hDUT. A. Expression of hDUT in *E. coli* BL21(DE3) cells. M: molecular mass marker, -I: before induction, +I: after induction. B. Purification of hDUT. M: molecular mass marker, 1: supernatant after centrifuging the cell lysate, 2: resuspended precipitate cell lysate, 3: before anion exchange column, 4: after anion exchange column, 5: after gel filtration. C. Expression of His-hDUT in *E. coli* BL21(DE3) (pLysS) cells. M: molecular mass marker, -I: before induction, +I: after induction. D. Purification of His-hDUT. M: molecular mass marker, 1: supernatant after centrifuging the cell lysate, 2: resuspended precipitate cell lysate, 3: flowthrough of the NiNTA column, 4: salty washing step, 5: 50 mM imidazole washing step, 6: elution of His-hDUT by 0.5 M imidazole. This fraction was subsequently dialysed into the appropriate buffer.

4.2 Human dUTPase

4.2.1 Analysis of human dUTPase crystal structure (ref. 1 in the Publication List)

Before the present studies, there had been two crystal structures available in the protein data bank, human dUTPase in its apo form (PDB ID: 1Q5U), and in complex with dUDP (PDB ID: 1Q5H). The crystal structure of human dUTPase in complex with α,β-imido-dUTP and Mg$^{2+}$ at a resolution of 2.2 Å was determined in our laboratory, and was deposited in the PDB (PDB ID: 2HQU). This crystal structure shows the same overall protein fold and secondary structure (cf. 1.2) as the previous ones (Figure 6. A). There are, however, novel features in this structure as compared to both earlier deposited structures: the full-length C-terminal region is visualized for two monomers, and the structure contains an isosteric
substrate analogue [56] in the catalytically competent gauche conformation, as well as the cofactor Mg$^{2+}$ (Figure 6. B) [3, 57].

Figure 6. 3D structure of the human dUTPase:α,β-imido-dUTP:Mg$^{2+}$ complex. Colour-coded ribbon models of the subunits are presented with ligand molecules and Mg$^{2+}$ shown as ball-and-stick model (yellow carbons and otherwise atomic colouring). A. Overall 3D structure. B. Close-up of the interactions formed by Arg153. C. Interactions of the C-terminal tail with the ligand molecule. The C-terminal segment of monomer A is shown, with residues and ligand as ball-and-stick model. Ligand carbons are in yellow, protein carbons according to subunit colours, other atoms in atomic colouring. Rest of the protein is presented with ribbon model and transparent surface of the protein core. H-bonding interactions are in dashed lines. Note the extensive number of interactions formed between the C-terminus and the phosphate chain of the ligand that is in contrast to the limited number of interactions formed with the dUMP moiety. D. β-turns accommodating the phosphate chain of the ligand, graphic representation as in panel C.

Mg$^{2+}$ forms a tridentate complex with one oxygen from each of the three phosphate groups of the nucleotide, and also coordinates three water molecules, similarly to the previous report on *E. coli* dUTPase [3] (Figure 6. B). Both the only structure having the entire C-terminus visualized (dUTPase from *feline immunodeficiency virus* [7]) and the single liganded human dUTPase structure [8] lacked the nucleotide γ-phosphate group and the co-factor Mg$^{2+}$ and contained the α-phosphate in the trans configuration, unavailable for nucleophilic attack.
Visualisation of the catalytically competent conformation (including Mg\(^{2+}\)) together with the entire C-terminus reveals the important interaction pattern of the C-terminus, which could only be hypothesised previously. There are only two interactions between the C-terminal tail and the dUMP moiety. The first one is the Phe158 ring that stacks over the uracil ring, and the second one is the Phe158 amide N that H-bonds with the deoxyribose ring (Figure 6. C). In contrast, numerous contacts link the pyrophosphate leaving group to the C-terminus, suggesting its preferred localization upon substrate binding. Main chain atoms of residues Gly159–Thr161 form H-bonds to the \(\beta\)- and \(\gamma\)-phosphates. Arg153 contributes to neutralization of the \(\gamma\)-phosphate negative charge, in agreement with its essential role [58]. Arg153 also forms H-bonding with Ser42 of Motif 2 from the other subunit (Figure 6. B); this interaction helps to localize Arg153 to the core of the protein. The guanidino group approaches the main chain Gly156 O atom to form a small loop that may direct the flexible Arg side chain for functional interactions with the phosphates. An additional H-bond between Gly156 main chain N and Arg153 main chain O stabilizes this loop in an appropriate conformation (Figure 6. C). For the first time, the role of the conserved Ser160 and Thr161 residues, suggested to be of importance [45], could be assigned (Figure 6. C). The hydroxyl groups of these residues form H-bonds with the two oxygen atoms of \(\gamma\)-phosphate not involved in chelating Mg\(^{2+}\). Interestingly, Thr161 and Gly162 from one monomer also contact the side chains of Arg85 and Arg128, the conserved arginines from Motifs 2 and 4 from the neighbouring monomer involved in the active site. These interactions contribute to a fine-tuned charge distribution around the active site and also link the C-terminus of one monomer to residues in the other monomer within the core of the trimer. The Ser160N–Gly157O and Gly159O–Gly162N interactions shape two consecutive Type I turns for \(\gamma\)-phosphate accommodation (Figure 6. D). The many contacts of the pyrophosphate group with the C-terminus are in contrast with its few interactions with the core of the trimer (H-bonding to an Arg85 side chain and to Gly87 main chain N). Mg\(^{2+}\) is tightly bound to the phosphate chain, but contacts protein residues only indirectly via water molecules. These data suggest that the pyrophosphate:Mg\(^{2+}\) complex could leave the active site with support from the flexible C-terminus. Ordering of the C-terminal arms is not equivalent within the three monomers: two of these arms are associated with appreciable electron densities (‘closed’ active sites), while the third one cannot be localized. Similar asymmetry was already observed (PDB ID:1Q5U [7]). Detailed analysis of crystal packing interactions did not reveal any difference that may
have resulted in this asymmetry. The distance between the attacking nucleophilic water oxygen and the substrate α-P atom (2.77 and 2.79 Å) in the two ‘closed’ active sites show the approach of the reactant atoms during catalysis. (While it is 3.23 Å for the ‘open’ active site.) The sum of van der Waals radii for oxygen and phosphorus is 3.32 Å, i.e. significantly larger than observed in the closed active sites. Therefore, the presently determined crystal structure of human dUTPase visualises a novel structure that may represent a near-attack conformation. In this structure, approach to the intermediate of the reaction that is initiated by the nucleophilic attack of the incoming water oxygen atom on the α-P is directly observed for the first time.

4.2.2. Structural analysis in solution (ref. 1 in the Publication List)

Investigation of protein structure by X-ray crystallography allows the determination of high-resolution three dimensional structures where atoms can be localised in the 3D space with great certainty. However, these observations are inherently static, and dynamics of protein conformations cannot be fully determined. Solution phase experiments are therefore crucial for full assessment of protein structure and function characteristics.

4.2.2.1 Thermal stability

In order to gain information about the folding and stability of the recombinant protein in solution, differential scanning calorimetry was applied [46, 59]. Melting temperature was found to be 61.7°C (Figure 8. A), similar to the values obtained for Drosophila melanogaster or Plasmodium falciparum dUTPases (56.5°C and 63.8°C, respectively) and considerably lower than for E. coli dUTPase (74.7°C), indicating decreased stability of eukaryotic dUTPases [59].

Lower thermostability may be partially due to increased flexibility of the N- and C-terminal segments. In fact, the N-terminus could not be localized in the 3D structure obtained from our recombinant protein, and for one monomer, the C-terminus was also missing from the density maps. To analyse conformational shifts of these flexible segments possibly induced by substrate/product binding, limited proteolysis experiments were performed.
Limited proteolysis provides information on flexible segments. Human dUTPase was, therefore, trypsinolysed in three parallel experiments, under different circumstances, in order to investigate any putative substrate/product induced conformational changes that might affect the overall fold and thus are detectable by an altered sensitivity towards trypsic digestion. In the first experiment, the apoenzyme was subjected to limited trypsinolysis (i.e. no ligand was present in the solution), in the second one the digestion was carried out in the presence of the product, dUMP (5 mM), while in the third one in the presence of the substrate analogue, α,β-imido-dUTP (0.5 mM).

Time course of proteolysis was followed by SDS-PAGE (Figure 7. A, B, C, respectively). Gel bands were cut and subjected to mass spectrometry. Three trypsic sites have been identified this way. Masses at MH⁺=16131±3 and 15181±3 correspond to fragments I [16–164] and II [16–153], respectively, so the two main trypsic cleavage sites occur at Arg15 and Arg153. N-terminal microsequencing of these fragments confirmed cleavage site at Arg15 with Ala-Arg-Pro-Ala-Glu starting sequence. Tryptic cleavage at this site is completed in less than five minutes regardless of the presence or absence of any nucleotides, and was also observed to occur spontaneously in stock solutions (for the non-tagged species especially; as for the His-tagged recombinant proteins, this phenomenon was typically non-observable) suggesting the flexibility of the N-terminal segment and indicating that neither does it form any significant interactions with the nucleotides nor does it undergo any significant conformational rearrangements upon nucleotide binding. However, binding of α,β-imido-dUTP just as well as dUMP exerts a significant protection against cleavage at Arg153. The third trypsic site has only been identified in the apoenzyme probably at Arg44 (fragment III [44-153]). This residue is located close to the nucleotide binding pocket; ligand binding may therefore induce a local subtle conformational change around this region resulting in protection against trypsinolysis.

Time course of proteolysis was also followed by enzyme activity measurements in parallel to gel sampling (Figure 7. D). The observable loss of enzymatic activity followed an apparent first order reaction with rate constants 0.0260, 0.0138 and 0.0023 min⁻¹ for human dUTPase in the absence of any nucleotides, in the presence of dUMP, and in the presence of α,β-imido-dUTP, respectively, in agreement with the rate estimated from MS results for
cleavage at Arg153. Cleavage at this site results in the loss of the major part of the C-terminal arm including most of the 5th highly conserved motif. In addition to this, remaining activity is in good correlation with the amount of fragment I [16–164], indicating that the C-terminus is essential for activity. (Since no significant decrease of dUTPase catalytic activity is observable after the cleavage of segment [2-15], results of the limited proteolysis experiment suggest this first fragment is very unlikely to have any role in catalysis.) The protective effect of the substrate analogue is more pronounced arguing for a different enzyme conformation in complex with its substrate and its product (Figure 7. B and C).
Figure 7. Domain analysis by limited trypsinolysis of human dUTPase. A. In the absence of nucleotide ligands at time points 0, 5, 30, 60 minutes. Roman numerals refer to human dUTPase peptide fragments resulted from the limited trypsinolysis: fragment I [16-164], fragment II [16-153] and fragment III [44-153] B. In the presence of the product, dUMP at time points 0, 5, 120, 240 minutes C. In the presence of the substrate analogue, α,β-imido-dUTP at time points 0, 5, 120, 240 minutes. MM: molecular mass marker in all three cases with the relative molecular masses indicated. D. Time course of trypsinolysis followed by enzyme activity measurements. Graphs show loss of enzymatic activity due to an apparent first order reaction with rate constants shown in Table III. The curves of the digestion in the absence of any nucleotides, in the presence of dUMP and in the presence of α,β-imido-dUTP are indicated with straight line, dashed line and dotted line, respectively. E. Primary structure of the nuclear isoform of human dUTPase. The five highly conserved motifs are boxed. The fragments identified by mass spectrometry are in bold face. Grey background indicates the five residues of the first two tryptic fragments (I and II) as identified by N-terminal microsequencing. The three subsequent tryptic sites are marked with arrows. The straight and dashed crosses represent the complete and partial protection exerted by the two nucleotides.
Table 2. Comparison of the applied methods in the limited tryptic digestion of different dUTPases.

<table>
<thead>
<tr>
<th></th>
<th><em>E. coli</em> dUTPase [15, 49]</th>
<th><em>D. mel.</em> dUTPase [46]</th>
<th>Human dUTPase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzyme concentration</strong></td>
<td>125 μM, 2 mg/ml</td>
<td>29 μM, 0.5 mg/ml</td>
<td>28 μM, 0.5 mg/ml</td>
</tr>
<tr>
<td><strong>Trypsin concentration</strong></td>
<td>200 μg/ml</td>
<td>10 μg/ml</td>
<td>2 μg/ml</td>
</tr>
<tr>
<td><strong>Trypsin/dUTPase mass ratio</strong></td>
<td>1:10</td>
<td>1:50</td>
<td>1:250</td>
</tr>
<tr>
<td>$k_{app}$ (when no ligand is present)</td>
<td>0.0380 min⁻¹</td>
<td>0.0096 min⁻¹</td>
<td>0.0260 min⁻¹</td>
</tr>
<tr>
<td>$k_{app}$ (in the presence of dUMP)</td>
<td>not determined</td>
<td>0.0048 min⁻¹</td>
<td>0.0138 min⁻¹</td>
</tr>
<tr>
<td>$k_{app}$ (in the presence of $\alpha,\beta$-imido-dUTP)</td>
<td>0.0082 min⁻¹</td>
<td>0.0013 min⁻¹</td>
<td>0.0023 min⁻¹</td>
</tr>
</tbody>
</table>

When comparing proteolytic studies on different dUTPase homotrimers (human, *E. coli* [15] and *D. mel.* dUTPases [46]), an increased sensitivity of the eukaryotic enzymes is observable (Table 2). The human enzyme appears to be the most sensitive one against trypsinolysis suggesting a relatively increased flexibility of hDUT as compared to these other dUTPases. Increased flexibility of eukaryotic vs prokaryotic dUTPases is also supported by the differential scanning calorimetry results discussed above (see Figure 8. A, below) (c.f. also [59]).

An additional difference between the limited trypsinolysis of pro- and eukaryotic dUTPases is the extent of the protective effect of $\alpha,\beta$-imido-dUTP. It is larger in the eukaryotic enzymes than in *E. coli* dUTPase arguing for a more pronounced conformational shift upon substrate binding.
4.2.3 Equilibrium binding studies (ref. 1 in the Publication List)

4.2.3.1 Isothermal calorimetry

In order to determine the binding constant of $\alpha,\beta$-imido-dUTP, isothermal titration calorimetry was performed as described in Materials and Methods. The plot of isothermal titration of hDUT with $\alpha,\beta$-imido-dUTP is shown in Figure 8. B. In an attempt to gain quantitative information about the binding profile of $\alpha,\beta$-imido-dUTP to human dUTPase based on the isothermal titration calorimetric binding isotherm, the different built-in models of the isothermal titration calorimetry, namely the one type of sites, the two sets of sites, and the sequential binding sites model, were fitted to the experimental data (cf. Materials & Methods). Out of the three models, the two sets of sites and the sequential binding sites model gave similar good fits ($\chi^2 = 1968$, and $\chi^2 = 2079$ for the former and the latter models, respectively) while the one type of sites model was the least close to the experimental data ($\chi^2 = 16345$). This result, together with that of the limited proteolysis indicated a fine-tuned regulation of the three binding sites. However, all the experiments described above were carried out in phosphate buffer, and based on a recent study of dCTP deaminase from *E. coli*, where false cooperativity was detected directly correlating with phosphate (buffer) concentration [60], the same isothermal titration was carried out in HEPES buffer (Figure 8. C). The $\chi^2$ values of the different fitted models were not very different from each other (15780 for the one type of sites model, 9154 for the two sets of sites and 9674 for the sequential binding sites model), thus not supporting the assumption of allostery/cooperativity in substrate binding. Later on, the same titration was repeated at 20°C to have a binding isotherm at the same temperature as the fluorescent titrations (to be discussed in chapter 4.2.3.2), but this one was similar to the previous isotherm measured in HEPES buffer (Figure 8. C and D). Dissociation constant of the dUTPase:$\alpha,\beta$-imido-dUTP complex was determined to be 7.2 ± 0.2 $\mu$M from the latest isotherm, which value is very similar for homologous enzymes [49, 58].
Figure 8. Summary of calorimetric measurements. A. Heat induced denaturation of human dUTPase followed by differential scanning microcalorimetry. Melting temperature was determined to be 61.7°C (as shown in the figure). B. Titration of dUTPase with α,β-imido-dUTP followed by isothermal calorimetry in phosphate buffer at 28°C. The one type of sites model fitting is shown in the figure. Due to the high $\chi^2$ (16345), the parameters are not reliable: $n = 3.02 \pm 0.01$, and $K_d = 11.8 \pm 0.4 \mu M$. C. Titration of dUTPase with α,β-imido-dUTP followed by isothermal calorimetry in HEPES buffer at 28°C. The one type of sites model fitting is shown in the figure. $K_d$ was estimated to be $15 \pm 0.5 \mu M$ (as indicated in the figure). D. Titration of dUTPase with α,β-imido-dUTP followed by isothermal calorimetry in HEPES buffer at 20°C. The one type of sites model fitting is shown in the figure. $K_d$ was estimated to be $7.2 \pm 0.2 \mu M$ (as indicated in the figure).

Even though the isothermal titration calorimetry measurements enabled the determination of the binding constant of α,β-imido-dUTP, an independent method was sought to examine the question of phosphate effect and to determine the product binding profile.
Additionally, a sensitive system capable of reporting about stoichiometric ligand binding was desirable.

4.2.3.2 Fluorimetry

As described previously, Phe158 in human dUTPase makes an interaction with the uracil ring. A mutant of His-hDUT was designed by the replacement of this Phe158 by Trp, and thus a highly sensitive fluorescent label within the C-terminus was obtained, with no apparent change in $k_{cat}$ (as described in section 4.2.4.3). Fortunately, no other Trp residues are present in the hDUT protein. The saturation curve for both the substrate analogue and the product were obtained, and the quadratic equation with 1:1 stoichiometry for each subunit was fitted to the experimental data:

$$y = s + A[(c+K+x)-((c+K+x)^2-4cx)^{1/2}]/(2c)$$

where $x$: nucleotide concentration; $y$: fluorescent intensity; $s$: intercept; $A$: amplitude; $c$: enzyme concentration; $K$: $K_d$.

$K_d$ values were found to be 1.6 ± 0.2 μM for $\alpha,\beta$-imido-dUTP and 26.3 ± 2.8 μM for dUMP. Trp quenching is significantly larger in the dUTPase: $\alpha,\beta$-imido-dUTP complex (approx. 60%) than in the dUTPase:dUMP complex (approx. 45%) (Figure 9. A), arguing for different C-terminus conformations in the two nucleotide binding states, in agreement with the trypsinolysis results. Binding strength of the substrate analogue to the human enzyme is very similar to the *E. coli* protein, but product binding is much stronger in the human enzyme than in the bacterial protein (cf. [53]).

Figure 9. C shows His-hDUT$^{158W}$ - $\alpha,\beta$-imido-dUTP saturation curves in different buffers (the same experiments with the product yielded similar results - data not shown). Apparently, there is no large difference in the binding/saturation profile of either the substrate analogue or the product in different buffers. To determine the nucleotide binding profile, that is if any allostery can be detected, the Hill equation ($y = V_{max} * x^n/(k^n + x^n)$, where $V_{max}$: signal at saturating nucleotide concentrations, $x$: nucleotide concentration, $k$: $K_d$, and $n$: Hill coefficient) was fitted to the His-hDUT$^{158W}$ - nucleotide saturation curves in both buffers (Figure 9. B and C). The Hill coefficient, $n = [0; N_b]$, where $N_b$ = the number of binding sites (3 for dUTPases). When $n$ = the number of binding sites, the system behaves as perfectly
cooperative, whereas \( n = 1 \) indicates no cooperativity, while \( 0 < n < 1 \) suggests negative cooperativity.

![Fluorescence spectra of unliganded His-hDUT\(^{158W}\) (straight line), His-hDUT\(^{158W}\)-dUMP complex (dashed line) and His-hDUT\(^{158W}\)-α,β-imido-dUTP complex (dash dotted line) B. Saturation curve of α,β-imido-dUTP in TrisHCl buffer. The Hill equation (\( y = V_{\text{max}} \times x^n/(k^n+x^n) \), where \( V_{\text{max}} \) signal at saturating nucleotide concentrations, \( x \) nucleotide concentration, \( k \) \( K_d \), and \( n \) Hill coefficient) fitting is shown in the figure. C. Saturation curve of α,β-imido-dUTP in TrisHCl buffer (square) and in NaPi buffer (triangle). The Hill equation fitting is shown in the figure. D. Saturation curve of dUMP in TrisHCl buffer. The Hill equation fitting is shown in the figure.

Figure 9. Equilibrium ligand binding followed by fluorimetry. A. Fluorescent spectra of unliganded His-hDUT\(^{158W}\) (straight line), His-hDUT\(^{158W}\)-dUMP complex (dashed line) and His-hDUT\(^{158W}\)-α,β-imido-dUTP complex (dash dotted line) B. Saturation curve of α,β-imido-dUTP in TrisHCl buffer. The Hill equation (\( y = V_{\text{max}} \times x^n/(k^n+x^n) \), where \( V_{\text{max}} \) signal at saturating nucleotide concentrations, \( x \) nucleotide concentration, \( k \) \( K_d \), and \( n \) Hill coefficient) fitting is shown in the figure. C. Saturation curve of α,β-imido-dUTP in TrisHCl buffer (square) and in NaPi buffer (triangle). The Hill equation fitting is shown in the figure. D. Saturation curve of dUMP in TrisHCl buffer. The Hill equation fitting is shown in the figure.

The results summarised in Figure 9. show that both the substrate analogue and the product binding profile clearly lacks any cooperative character, regardless of the buffer.

The \( K_d \) values of α,β-imido-dUTP experimentally determined by isothermal titration calorimetry and fluorimetry are comparable with each other, and both indicate the high affinity of human dUTPase towards its substrate.
4.2.4 Kinetic analysis (ref. 2 in the Publication List)

4.2.4.1 Steady-state activity

The ‘conventional dUTPase assay’ generally used to follow the reaction uses the phenol red pH indicator to detect proton release continuously as described in [53] (Figure 10.). The catalytic reaction can be written as follows: dUTP $\rightarrow$ dUMP $+$ PP$_i$ $+$ nH$^+$, where n depends on ambient pH, and at pH = 7.5, it was found to be 1 [61].

This assay was used to determine the $k_{cat}$ value of hDUT, which was found to be $4.8 - 6.4$ s$^{-1}$. The $k_{cat}$ values of His-hDUT and His-hDUT$^{158W}$ determined with this assay gave the same results indicating the replacement of Phe158 by Trp does not alter the catalytic activity. The $k_{cat}$ value of His-hDUT$^{158W,102N}$ was determined to be approx. $4 \cdot 10^{-3}$ s$^{-1}$, that is at least three orders of magnitude less than for the wild-type enzyme.

![Figure 10. A typical progress curve of the dUTPase reaction, as measured by the phenol red assay.](image)

4.2.4.2 An attempt to determine $K_M$

This phenol red assay was used when we first tried to determine $K_M$ by producing a $v_0$ vs. substrate concentration plot. Spectrophotometric experiments failed to reveal the true
nature of this correlation due to the high error rate, even though signal – concentration linearity was provided (Figure 11. A and B).

Figure 11. Summary of the $v_0$ vs dUTP concentration curves. A. Spectrophotometric detection. Data are averages of five parallel measurements. High errors indicate that spectrophotometric detection using this assay is not sensitive enough to yield the desired plot. B. Signal (absorbance change of phenol red upon protonation at 559 nm) change appears to be linear in the range, where plot A was measured. C. The same relation tested in the stopped-flow instrument. This setup enabled the measurement of lower dUTP concentrations resulting in the appearance of the initial phase of the curve. It also shows a decrease at higher dUTP concentrations, in agreement with the spectrophotometric results. D. Initial velocity vs dUTP concentration in the case of E. coli dUTPase. The Hill-plot was fitted to the experimental points. $K_M$ was found to be $0.80 \pm 0.13 \, \mu M$, $n = 1.1 \pm 0.2$.

The same experimental setup was also used in the stopped-flow instrument, but the sensitivity was too low for stopped-flow detection. The setup was optimised in order to decrease response time (see Transient kinetics by stopped-flow section of Materials and Methods). This setup was sensitive enough to determine the initial velocities of much lower dUTP concentrations than in the spectrophotometer, so the initial increasing phase of the $v_0$
vs. substrate concentration plot became visible (Figure 11. C). It was even possible to reproduce the available data on *E. coli* dUTPase (Figure 11. D). However, error rates were still high (not indicated), and the human dUTPase experiments yielded similar results to those of the spectrophotometric assay showing a marked decrease at higher substrate concentrations. The reason for this behaviour is still unclear, and needs further investigations.

Since the conventional dUTPase assay did not provide the information desired, the tryptophan signal of His-hDUT$^{158W}$ was followed during the catalytic reaction.

4.2.4.3 Resolving the catalytic mechanism: transient kinetics

A complete progress curve was recorded in the stopped-flow instrument as described in the Materials & Methods section. The progress curve appeared to be suprisingly complex, as shown on Figure 12. A. After a very rapid initial step, probably reflecting dUTP binding, there was a phase having an apparently different fluorescent signal from that of the referring hDUT:α,β-imido-dUTP complex. To investigate if this state was really different in the case of the real substrate and the substrate analogue, the inactive His-hDUT$^{158W,102N}$ was designed. Asp102 is a highly conserved residue in Motif 3, which has a crucial role in catalysis by coordinating the nucleophilic water molecule towards the α phosphorus atom. In agreement with its essential role, the replacement of this residue by Asn deteriorates the fine-tuned charge distribution in the active site resulting in the loss of activity, as demonstrated by activity measurements. However, since it does not appear to have any role in coordinating the substrate, there was a chance to detect the difference in the binding of the substrate and its analogue using the intrinsic Trp sensor. Figure 12. B shows the titration of His-hDUT$^{158W}$ with α,β-imido-dUTP, the titration of His-hDUT$^{158W,102N}$ with α,β-imido-dUTP and the titration of His-hDUT$^{158W,102N}$ with dUTP. The inactive mutant enzyme apparently has the same binding profile with the substrate as the active one has with the substrate analogue, while α,β-imido-dUTP causes smaller Trp quenching in the inactive enzyme. The results clearly show the difference between the binding of the substrate and its analogue to the inactive enzyme indicating the same could apply to the wild-type enzyme as well.
Figure 12. The signal of the intrinsic Trp sensor. A. Progress curve of the dUTPase reaction. The later identified steps are the first rapid binding, a subsequent isomerisation step, the rate limiting hydrolysis step, and the rapid, nonordered release of the products. B. The built-in Trp fluorescence quenching of His-hDUT^{158W} or hDUT^{158W,102N} by saturation with different nucleotides. 3 titration curves are plotted to demonstrate the substrate and its analogue have different binding conformations. Squares represent His-hDUT^{158W} vs α,β-imido-dUTP, diamonds represent His-hDUT^{158W,102N} vs dUTP and triangles represent His-hDUT^{158W,102N} vs α,β-imido-dUTP saturation curves. C. Fluorescence emission spectra of hDUT^{158W} at saturating concentrations of ligands. Data are normalized to the emission peak of the apo enzyme. [hDUT^{158W}] = 4 μM, [dUMP] = 500 μM, [dUDP] = 300 μM, [α,β-imido-dUTP] = 100 μM, [dUTP] = 2 mM, [PPi] = 5 mM. To capture the dUTP-bound cycling steady state, a high excess of dUTP was used, and the spectrum was recorded within 30 s after dUTP was added.

In order to find the reason for the different fluorescent states of the two enzyme:nucleotide complexes and to carry out a detailed analysis, the maximal fluorescence changes and spectral shifts of Trp158 upon binding to physiological ligands and to the nonhydrolyzable substrate analogue were quantified (Table 3).
Table 3. Fluorescence properties of hDUT<sup>158W</sup> apoenzyme and its ligand-bound complexes

<table>
<thead>
<tr>
<th>ligand</th>
<th>K&lt;sub&gt;d&lt;/sub&gt; (μM)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
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</thead>
<tbody>
<tr>
<td>none</td>
<td>-</td>
<td>353</td>
</tr>
<tr>
<td>dUMP</td>
<td>32 ± 2</td>
<td>347</td>
</tr>
<tr>
<td>dUDP</td>
<td>12 ± 1</td>
<td>347</td>
</tr>
<tr>
<td>dUTP</td>
<td>&lt; 1</td>
<td>339</td>
</tr>
<tr>
<td>α,β-imido-dUTP</td>
<td>5 ± 3</td>
<td>343</td>
</tr>
<tr>
<td>dUMP.PPi</td>
<td>479 ± 20</td>
<td>351</td>
</tr>
<tr>
<td>PP&lt;sub&gt;i&lt;/sub&gt;</td>
<td>146 ± 15</td>
<td>351</td>
</tr>
</tbody>
</table>

These data were recorded similarly to those described above (cf. section 4.2.3.4), and yield information on the interaction of Trp158 with the uracil moiety of any bound nucleotide and will allow interpreting the fluorescence-based kinetic experiments. The Trp158 fluorescence emission maximum of the apoenzyme was at 353 nm, a typical value for a nonburied protein tryptophan (Figure 12. C). Figure 12. C also shows that the binding of different uracil nucleotides but not that of PP<sub>i</sub> to His-hDUT<sup>158W</sup> quenches Trp158 fluorescence, probably due to aromatic stacking between the indole and uracil rings (shortest distances between atoms of the uracil moiety and those of the Phe158 benzene ring in hDUT are 3.4 –3.7 Å, as determined in the crystal structure. The magnitude of the nucleotide-induced quench and blue shift increased in the order dUMP→dUDP→α,β-imido-dUTP. This implies that the presence of the β- and γ-phosphates causes the C-terminal arm to form more interactions with the phosphate chain of the substrate (in agreement with the structural description), whereby the arm may become less flexible and may stabilize the stacking interaction between Trp158 and the uracil ring. Interestingly, Trp158 fluorescence was even more quenched during steady-state dUTPase cycling than in any of the other ligand bound states (Figure 12. C). This suggests that there is at least one major steady-state intermediate that cannot be produced by the addition of the above ligands (e.g. the prehydrolysis mimic α,β-imido-dUTP or the posthydrolysis mimic dUMP.PPi states). A possible explanation for this finding is that a particular protein conformational change occurs in the presence of dUTP (but not in the presence of α,β-imido-dUTP or other nucleotides), leading to the hydrolysis-competent state (see below). The large fluorescence increase in the presence of PP<sub>i</sub> indicates that the binding of this ligand also causes a conformational change in the active site. The potential interaction of hDUT<sup>158W</sup> with phosphate (P<sub>i</sub>) (used at high excess) was also probed, but no signal change was detected.
$K_d$ values illustrate that the affinity of His-hDUT$^{158W}$ increases in the order dUMP→dUDP→α,β-imido-dUTP, with dUDP and α,β-imido-dUTP binding being 3 and 10 times stronger than that of dUMP, respectively. dUTP binding cannot be measured using this equilibrium method, but we anticipate that its $K_d$ value may be equal to or lower than that of α,β-imido-dUTP.

In the knowledge of the fluorescence characteristics of individual enzyme-substrate (substrate analogue) and enzyme-products complexes, progress curves obtained by monitoring Trp158 fluorescence during the interaction of His-hDUT$^{158W}$ with dUTP in the stopped-flow instrument yielded significantly more information than pH detection-based (proton release) methods. Both single and multiple dUTP turnovers were obtained using Trp158 fluorescence. Trp158 fluorescence traces of single dUTP turnovers ([E] > [S]) consisted of three exponential phases (Scheme 2.). A fast initial quench in fluorescence ($k_{obs} \sim 900 \text{ s}^{-1}$) was followed by an additional slower decrease ($k_{obs} \sim 20 \text{ s}^{-1}$), then the fluorescence intensity returned to a close-to-initial value with a $k_{obs}$ of $6.8 \pm 2.0 \text{ s}^{-1}$. In the light of the steady-state fluorescence data of Figure 12. C, we interpret the first fast phase as the initial binding of the nucleotide in which Trp158 quenching occurs by stacking over the uracil ring. Considering the difference between the fluorescence intensity of the enzyme:α,β-imido-dUTP complex and that during steady-state dUTPase cycling (and in agreement with the findings of the inactive enzyme fluorescent titrations), the second slower phase can be interpreted as a dUTP-induced structural change that precedes or is concomitant with dUTP hydrolysis. The third phase reflects the slowest rate-limiting step of the cycle (dUTP hydrolysis or product release). (The identities of the steps associated with the second and third phases were clarified in quenched-flow and PP$_i$ chasing experiments, cf. ref. 2 in the Publication List).

Determination of the substrate concentration dependence of the rate constant of the first phase under pseudo first order conditions was challenging because either the signal to noise ratio was too low for reasonable resolution (when attempting to decrease [S] at a constant [E] (maintaining [S] << [E])), or the amplitude became completely lost in the dead-time of the stopped-flow apparatus (when applying a several-fold excess of [S] over the lowest detectable [E]). Measurements carried out using near-equimolar concentrations of enzyme and substrate indicated that the time course of this phase does depend on concentration ($k_{obs}$ values of force-fitted exponentials were 400-1200 s$^{-1}$ in the applied 2.5-15 μM concentration range). Numerical simulations in which this phase was assigned to a second-order binding step
showed good agreement with the experimental traces and the fundamental rate constants could be extracted (cf. Scheme 2, below). We did not observe systematic concentration dependence of the $k_{\text{obs}}$ (termed $k_{\text{ISO,obs}}$) of the second exponential phase ($20 \pm 18 \text{s}^{-1}$), which confirms the first order nature of this proposed isomerisation step. The $k_{\text{obs}}$ of the third phase did not exhibit concentration dependence in the single turnover concentration regime. The $k_{\text{obs}}$ of this phase was in good agreement with the previously determined steady-state $k_{\text{cat}}$ of hDUT ($8 \pm 3 \text{s}^{-1}$), indicating that it represents the rate-limiting step of the dUTPase cycle. Furthermore, the duration of the steady-state ($t_{\text{ss}}$) in multiple turnover Trp158 fluorescence traces (i.e. the time elapsed between the start of the reaction and the inflection point of the fluorescence restoration phase, Figure 12. A) was consistent with the above third phase $k_{\text{obs}}$ and steady-state $k_{\text{cat}}$ values ($t_{\text{ss}} \approx [S]_{\text{initial}}/([E]_{\text{total}}k_{\text{cat}})$, if $[S]_{\text{initial}} \gg K_M$).

Global fits to the single and multiple turnover time courses yielded $K_M = 3.6 \pm 1.9 \mu\text{M}$, $k_{\text{cat}} = 6.7 \pm 0.2 \text{s}^{-1}$, $k_{\text{cat}}/K_M \sim 1.9 \times 10^6 \text{M}^{-1}\text{s}^{-1}$, for both His-hDUT and His-hDUT$^{158W}$ proteins.

Our conclusion is that the dUTP hydrolysis cycle consists of at least four distinct enzymatic steps: (i) fast substrate binding, (ii) isomerization of the enzyme-substrate complex into the catalytically competent conformation, (iii) a hydrolysis (chemical) step, and (iv) rapid, nonordered release of the products. Independent quenched-flow experiments indicate that the chemical step is the rate-limiting step of the enzymatic cycle.
Scheme 2. Kinetic model of the hDUT enzymatic cycle. The graph of a time course upon mixing 7.5 μM His-hDUT\textsuperscript{158W} with 5.25 μM dUTP is shown, prepared for global fitting (fluorescence normalized to the apo enzyme, dead-time considered). The solid line is a global fit to the data points using the kinetic model shown in the scheme. Daggers and stars indicate fluorescence decrease or increase compared to the apoenzyme, respectively. The rate constants shown in the model were used as parameters of the kinetic simulation. For the $k_{PM}/k_{PM}$ and $k_{MP}/k_{MP}$ rate constant pairs, only the ratios and the lower bounds for the rate constant pairs are known. These lower bounds were used in the numerical simulations. Increases in the values of these rate constants (while keeping their respective ratios constant) did not cause any detectable change in the enzyme mechanism.
4.3 *Mycobacterium tuberculosis* dUTPase

4.3.1 Analysis of *M. tub.* dUTPase crystal structure (ref. 4 in the Publication List)

As the crystal structure of Chan et al. [9] (PDB ID 1MQ7) did not visualise the full C-terminal segment of the protein, we aimed at producing a *M. tub.* dUTPase crystal structure in which this segment is also visible and gives way to its analysis. Several conditions were tried, and finally the crystal structure of *M. tub.* dUTPase:α-β-imido-dUTP:Mg$^{2+}$ complex has been solved at 1.5 Å resolution (Figure 13. A) and deposited in the PDB (PDB ID: 2PY4). Even though this resolution is somewhat lower than that of Chan et al. (1.3 Å), the C-terminal arm with the species-specific hinge region has become visible in our structure for the first time. We analysed the interactions of the residues within this species-specific region with the other parts of the molecule, and also localised the His145 residue in *M. tub.* dUTPase replacing a highly conserved Phe. As described in the previous sections, this Phe residue has been shown to stack over the uracil ring of the substrate in both viral and human dUTPases. The polar replacement of this residue may challenge this role and may constitute a functional species-specific characteristic in *M. tub.* dUTPase. The His145 residue is situated next to the uracil ring of the substrate analogue in a manner much similar to the previously observed Phe-uracil stacking in human (cf. previous sections). In addition to van der Waals stacking interactions, polar character of this His residue also contributes to nucleotide accommodation. Both of the imino nitrogen atoms of the imidazole ring engage in water-mediated H-bonding; one is directed towards the O4 atom of the uracil ring, the other is towards the hydroxyl of Tyr86 residue that coordinates the deoxyribose ring (Figure 13. B). Interactions of the other species-specific structural element, the C-terminal tetrapeptide, that folds back and occupies a much complementary surface cavity of the neighbouring subunit, probably facilitate the ordering of Motif 5 upon the active site. Main chain H-bonds connect the Leu154, His151 and Gly146 amino acids. The Ala152 main chain imino nitrogen is H-bonded through another water to Asp109 side chain of the neighbouring subunit that is connected also to the His151 imidazole ring. Several van der Waals contacts are also observed: the Leu154 side chain contacts with the methylene groups of the Arg70 sidechain and the Leu67 side chain from the neighbouring subunit, while the Ser153 β-methylene is close to the Val71 γ-methylene (Figure 13. C).
The *M. tub.* specific five-residue loop (Glu132-Gly137) locally alters the peptide chain folding but residues within this segment do not show interactions either with the active site or with the protein surface (Figure 13. D).

Figure 13. Localization of the species-specific segments around the active site of *M. tub.* dUTPase. A. Main chain fold. The three α,β-imido-dUTP molecules (transparent spacefilling models with atom coded colouring (N, blue, O, red, P, orange, C, green)) are accommodated within the colour-coded subunit ribbon model. Peptide backbone of the species-specific segments Glu132–Ala136, His151-Leu154 as well as the *M. tub.* specific His145 are in magenta. B. Close-up of uracil-stacking. C. Interactions of the back-folded *M. tub.* C-terminal tetrapeptide His151-Leu154 with the protein surface of the neighbouring subunit. Dotted lines represent H-bonds and pink double arrows represent van der Waals interactions. D. Interactions of the *M. tub.* specific loop Glu132-Ala136.
4.3.2 Engineering a Trp sensor into *M. tub.* dUTPase (ref. 4 in the Publication List)

When searching for active site-binder inhibitors and especially for high-throughput-screening, a simple and sensitive reporter of the active site conformational changes is of great advantage. As demonstrated in the previous sections, the uracil-stacking Phe158 residue in human dUTPase proved to be such a reliable sensor. Since the structural analysis of *M. tub.* dUTPase revealed that the His145 residue also stacks over the uracil, the His145Trp mutant was constructed. This replacement successfully introduced the sensitive fluorescent label into the binding site, allowing for the detection of active site conformational changes upon the binding of both nucleotides and other types of molecules.

4.3.3 Testing the *M. tub.* dUTPase active site Trp sensor (ref. 4 in the Publication List)

Binding of nucleotides to the active site induces an intensity loss in the enzyme’s fluorescent signal (Figure 14. A), similarly to what was found for human dUTPase (cf. the referring section of human dUTPase). In order to test the tryptophan sensor and to determine the $K_d$ for the isosteric, non-hydrolysable substrate analogue (which value gives an upper limit for dUTP $K_d$, as described in the kinetic section 4.2.4.3), the enzyme was saturated with $\alpha,\beta$-imido-dUTP by titration followed by fluorimetry, and the quadratic equation with 1:1 stoichiometry for each subunit was fitted to the experimental data (cf. section 4.2.3.2):

$$y = s + A[(c+K+x)-((c+K+x)^2-4cx)^{1/2}]/(2c),$$

where $x$: nucleotide concentration; $y$: fluorescent intensity; $s$: intercept; $A$: amplitude; $c$: enzyme concentration; $K$: $K_d$ (Figure 14. B). *M. tub.* dUTPase binds the substrate analogue, $\alpha,\beta$-imido-dUTP with a $K_d = 0.17 \pm 0.05 \mu M$. This value is significantly different from that of human dUTPase ($K_d = 1.6 \mu M$), indicating that *M. tub.* dUTPase binds its substrate much stronger than the human enzyme. Based on the crystal structure data, the *M. tub.* specific His residue with its additional H-bonding and the more widespread interaction pattern between the C-terminus and the protein surface around the active site are proposed to be at least partially responsible for this strengthened binding.
4.3.4 Continuous activity assays (ref. 4 in the Publication List)

4.3.4.1 Phenol red assay

As described previously, the conventional phenol red indicator has so far been routinely used to follow the dUTPase reaction. The \( k_{\text{cat}} \) was determined with this assay and was found to be 3.7 – 5.8 s\(^{-1}\) for both His-mtDUT and His-mtDUT\(^{145W}\).

However, fluorimetric binding studies revealed that \( M. \text{tub.} \) dUTPase binds its substrate very strongly even as compared to the human enzyme, and thus the 40 µM dUTP concentration generally used in this assay is very much oversaturating. On the other hand, no reliable reaction traces can be obtained with substrate concentrations below 4 µM, due to the sensitivity limit of standard spectrophotometers. Moreover, the sensitivity of this assay to atmospheric CO\(_2\) absorption means the requirement of reproducibility (particularly at lower substrate concentrations) would be using inert atmosphere [53], which practically prevents its usage as a high-throughput screening assay. Thus, other methods were sought.
4.3.4.2 Coupled activity assay to detect dUMP formation

In search of a novel activity assay that would enable the continuous detection of the catalytic reaction, we first attempted to find a coupled enzymatic assay to follow dUMP formation. In order to do that, we tested if pyruvate kinase (E.C. 2.7.1.40) would act on dUMP as its substrate (since it does with dUDP cf. [46]), given the fact that this enzyme has a broad nucleotide specificity. If so, the pyruvate kinase – lactate dehydrogenase (E.C. 1.1.1.27) coupled enzyme reaction assay could have been optimised, using dUMP instead of ADP, as shown in Scheme 3. Upon the possible phosphorylation of dUMP into dUDP by pyruvate kinase, the absorbance change of the NADH → NAD⁺ transition in the subsequent reaction of NADH and pyruvate to NAD⁺ and lactate catalysed by lactate hydrogenase was intended to be followed spectrophotometrically at 340.

It turned out, however, that the introduction of dUMP into the reaction mixture instead of ADP does not induce any signal change, not even after more than 7 minutes, while the subsequent addition of ADP initiated the reaction immediately causing the completion of the reaction within the interruption time (Figure 15.). Thus, it can be concluded that pyruvate kinase does not recognise dUMP as a substrate.
Figure 15. Spectrophotometric detection of the NADH $\rightarrow$ NAD$^+$ transition at 340 nm. The first interruption marks the addition of dUMP to the reaction mixture, which clearly does not initiate any reaction. The proper set up was checked by the subsequent addition of ADP (second interruption), which proves the assay worked, but not with dUMP as a substrate of pyruvate kinase.

4.3.4.3 Coupled activity assay to detect pyrophosphate formation

The dUTPase reaction has another product: pyrophosphate. The next attempt was to find an assay capable of detecting pyrophosphate formation continuously. The commercially available pyrophosphate assay (EnzCheck Pyrophosphate Assay Kit) was first used, but was found to be useful only for end-point titration. So, in order that the catalytic reaction can be followed continuously, it had to be optimised on the basis of [54]. The optimised conditions were found (see Materials & Methods), and thus a coupled enzymatic activity assay was developed where the signal change is linear with product formation enabling the simple quantitative evaluation and comparison of progress curves recorded under different conditions (e.g. in the presence of different inhibitors). This is crucial for high-throughput screening, and even though this assay requires more materials than following the intrinsic Trp fluorescent signal, the relative material costs are decreased at larger scale, and the Trp signal was found to be quite complex, making the evaluation and particularly the comparison problematic. Thus, this assay provides a fast, sensitive and reliable continuous detection potential for even large-scale screening. Scheme 4. shows the coupled enzymatic reactions.
Scheme 4. The pyrophosphate coupled enzymatic reaction. In this case, the substrate of the second reaction is a modified purine nucleoside, namely the 2-amino-6-mercapto-7-methylpurine ribonucleoside (cf. [54]).

The optimised PPi assay was found to be much more sensitive than the phenol red assay; in the indicator assay 0.1 $\Delta A_{\text{total}}$ was observed by the hydrolysis of 40 $\mu$M dUTP, while the same 0.1 $\Delta A_{\text{total}}$ was observed by the hydrolysis of only 5 $\mu$M dUTP, when using the pyrophosphate assay (as shown in Figure 16.). This elevated sensitivity makes it possible to decrease the amount of dUTP hydrolysed to as low as 1 $\mu$M, since according to Webb, the limit of quantitative real-time phosphate detection is 2 $\mu$M. The $k_{\text{cat}}$ determined from this activity assay was found to be the same as the other one within error, reassuring the reliability of this novel coupled enzyme activity assay. Figure 16. shows the comparison of the progress curves of the phenol red and the pyrophosphate assays.

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The inhibitory effect of the substrate analogue at two different concentrations was also measured, in order to demonstrate the utility of this assay. Results are summarised in Table 4. It is interesting to note, that due to the tight binding of dUTP to dUTPase, the addition of α,β-imido-dUTP to the reaction mixture in the same concentration as the substrate (cf. Materials & Methods), decreases the initial velocity by only about 30% indicating that this enzyme may also bind its real substrate stronger than the substrate analogue.

Table 4. The inhibitory effect of α,β-imido-dUTP at two concentrations.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Relative Initial Velocity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>5 μM α,β-imido-dUTP</td>
<td>73.7 ± 8.9</td>
</tr>
<tr>
<td>50 μM α,β-imido-dUTP</td>
<td>40.1 ± 2.1</td>
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</table>
5. CONCLUSIONS

The analysis of both human and *Mycobacterium tuberculosis* dUTPase crystal structures revealed the important interactions of the C-terminal arm with the catalytically competent conformation of the substrate analogue and the cofactor Mg\(^{2+}\). It was found to make numerous interactions with the triphosphate moiety of the nucleotide while making only two contacts with the dUMP moiety. The comparison of these structures revealed that a species-specific 4 residue segment at the C-terminus right after the highly conserved motif 5 in *Mycobacterium tuberculosis* facilitates formation of the closed active site and may, at least partially, explain the tighter binding of substrate to *M. tub.* as compared to human dUTPase. It also became visible in the bacterial enzyme, that the His145 in Motif 5 replacing a conserved Phe in other dUTPases stacks over the uracil ring (like Phe in the human enzyme), despite its more polar character. Based on this finding, the replacement of this residue (His145 in *M. tub.* and Phe158 in *H. sap.* dUTPase) by Trp introduced a very sensitive intrinsic fluorescent sensor into the dUTPase active site.

Using the His-hDUT\(^{158W}\) mutant enzyme, both equilibrium binding studies and transient kinetic measurements could be carried out, which led to the determination of binding constants of both substrate analogue and product, and the complete quantitative description of the catalytic cycle. Binding of either the substrate or the product did not show any experimentally observable allosterism, but was found to follow a simple independent active site binding with 1:1 active site:nucleotide stoichiometry regardless of the buffer in agreement with isothermal titration calorimetry results. The determination of K\(_d\) values showed that the human enzyme binds its product much stronger than the *E. coli* enzyme, in agreement with our trypsinolysis studies. When comparing *E. coli* to human dUTPase, there is also a marked difference in the thermal stability indicating the higher flexibility, but lower stability of the eukaryotic enzyme, which is in agreement with studies on other eukaryotic dUTPases.

The human dUTPase catalytic cycle was found to consist of a rapid substrate binding, a subsequent isomerisation step during which the substrate becomes available for nucleophilic attack be a water molecule coordinated by the Asp102 residue, the rate limiting hydrolysis step, and the rapid, nonordered release of the products. This is the first study that could merge the structural data with the different catalytic steps giving an insight into the dUTPase catalytic mechanism and its structural background.
The significance of *M. tub.* dUTPase directed our investigations towards the potential of creating sensitive and reliable systems potent for high-throughput screening. One such system was the design of His-mtDUTH\(^{145W}\), that reports of even minor conformational changes in the active site, and so may be used for either testing active-site binders or to refine the search by measuring high-resolution kinetics. To provide a high-throughput assay, the pyrophosphate coupled assay was optimised yielding a robust continuous activity assay.
dUTPases catalyse the hydrolysis of dUTP into dUMP and pyrophosphate, being essential in maintaining DNA integrity this way. Both human and *Mycobacterium tuberculosis* dUTPases have been proposed as potential therapeutic targets. However, even though much information is available on the cellular level and regulation of physiological isoforms of the human enzyme, enzymological knowledge was still lagging behind.

To characterise the human enzyme, a high-yield expression system was created for the nuclear isoform of human dUTPase. Thermostability measurements and limited tryptic digestion revealed the elevated flexibility and decreased stability of the enzyme as compared to *E. coli* dUTPase. Detailed structural analysis revealed the important interactions of the C-terminal arm not visible before, and also identified the residues essential for catalysis.

Since Phe158 residue of highly conserved motif 5 stacks over the uracil ring of either the substrate or the product, this residue was mutated to Trp and binding studies were carried out using both this sensitive fluorescent sensor and isothermal titration calorimetry with α,β-imido-dUTP, a sterically faithful mimic of dUTP, to determine the *K*ₐ of both the substrate analogue and the product. This latter one was found to be significantly stronger than in the case of *E. coli* dUTPase.

This sensitive fluorescent label also enabled the first quantitative description of the human dUTPase catalytic cycle.

Studies on *Mycobacterium tuberculosis* dUTPase managed to identify species-specific segments in the enzyme structure for potential inhibitor targeting and to develop methods that enable the large scale screening of such inhibitors. A Trp sensor, similarly to the human enzyme, was introduced instead of His145, that replaces the otherwise conserved Phe residue. This sensor allows for the detection of active site binders and is a very sensitive reporter of active site conformational changes. The *K*ₐ of the substrate analogue was determined this way, and was found to be lower than for human dUTPase indicating the higher affinity of the bacterial enzyme towards the substrate. A robust coupled activity assay was also developed that might even be used for high-throughput screening.
7. ÖSSZEFoglalás

A dUTPázok a dUTP → dUMP + PPᵢ reakciót katalizálják, mely megelőző DNS javító szerepe miatt kiemelkedő fontosságú. Mind a humán, mind a Mycobacterium tuberculosis dUTPáz felmerült már lehetséges terápiás célpontként. Ilyen irányú vizsgálatokhoz azonban elengedhetetlen az enzim működésének alapos ismerete, melyet, a humán dUTPáz sejtbeli szabályozódásával ellentétben, még nem írtak le.

Ezen tanulmány egyik célja a humán dUTPáz jellemzőinek megismerése volt. A hőstabilitási és tripszinnel történő korlátozott hasítási kísérleteink kimutatták, hogy a humán enzim nagyobb flexibilitással, de kisebb stabilitással rendelkezik, mint az E. coli dUTPáz. Az enzim térszerkezetének elemzése feltárta a C-terminális kar aminosavainak a katalízis szempontjából kiemelkedő jelentőségű kölcsönhatásait a szubsztrát molekulával, ill. azonosítottuk a katalízisben döntő jelentőségű szerkezeti elemeket is.

Mivel a szigorúan megőrözött 5-ös motívum Phe158 aminosava átlapol a bekötődő nukleotid molekula uracil gyűrűjével, ezt az aminosavat lecsérélünk triptofánra, és kötődési vizsgálatokat végeztünk, mind az így létrejött mutáns enzim Trp fluoreszcenciáját követve, mind pedig izotermális titráló kalorimetriát végezve. Az eredmények azt mutatták, hogy a humán enzim erősebben köti a terméket, mint az E.coli dUTPáz.

Ezzel az érzékeny jellel lehetővé vált a katalitikus mechanizmus felderítése, valamint kvantitatív leírása is: Az első lépés a szubsztrát igen gyors bekötődése, melyet egy izomerizációs lépés követ. Ezután történik meg a kémiai lépés, azaz a hidrolízis, mely a sebesség meghatározó lépés. Ezt a termékek gyors, nem rendezett távozása követi. Ezekkel a vizsgálatokkal egyrészt először sikerült kvantitatívan leírni a dUTPáz katalitikus mechanizmusát, másrészt a mechanizmust a szerkezeti tulajdonságok fényében tudtuk magyarázni.

Az elemzésekben az uracil gyűrűvel átlapoló, a más fajokban megőrözött fenilalanint helyettesítő hisztidint lecsérélünk triptofánra. Így az aktív hely térszerkezeti változásait jól követő érzékelőt kaptunk. Emellett sikerült egy igen hatékony, a katalitikus reakciót folytonos módon követő aktivitás mérésere alkalmas kísérleti alkalmazást összeállítanunk, melynek áteresztőképessége elegendő nagyszámú gátlósz-jelölt vizsgálatára is.
REFERENCES


PUBLICATION LIST

My contribution to the publications

Active site closure facilitates juxtaposition of reactant atoms for initiation of catalysis by human dUTPase; FEBS Letters 581, 4783-4788 (2007):

I contributed to the analysis of the structural data, designed the His-hDUT and its mutant protein, carried out their cloning, optimised the expression and purification methods. I carried out the limited trypsinolysis experiments, the differential scanning microcalorimetry and – with the assistance of Dr. Ferenc Tölgyesi – the isothermal titration calorimetry. I also did the fluorescent titrations, as well as all the data processions of the solution phase experiments (for the isothermal titration calorimetry with Dr. Ferenc Tölgyesi). I also contributed to the interpretation of the results as well as writing the manuscript.


I contributed to the stopped-flow experiments, both with the absorbance and fluorescence detection, and the equilibrium binding studies.

Experimental study on dUTPase-inhibitor candidate and dUTPase/disaccharide mixtures by PCS and ENS; Journal of Molecular Structure, in press:

I contributed to the referring parts (i.e. His-mtDUT protein expression and purification).

Active site of mycobacterial dUTPase: structural characteristics and a built-in sensor; BBRC accepted:

My contribution was the test of the tryptophan sensor, the equilibrium binding studies, the optimisation of all the activity assays, and interpretation of the results as well as taking part in writing the manuscript.

Publications related to the present thesis

Journal articles


3.) Balázs Varga, Federica Migliardo, Enikő Takács, Beáta G. Vértessey, Salvatore Magazù: Experimental study on dUTPase-inhibitor candidate and dUTPase/disaccharide mixtures by PCS and ENS; Journal of Molecular Structure, in press.

4.) Balázs Varga, Orsolya Barabás, Enikő Takács, Nikolett Nagy, Péter Nagy, Beáta G. Vértessey: Active site of mycobacterial dUTPase: structural characteristics and a built-in sensor; BBRC accepted.

Conference proceedings


Other publications

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