

Ph.D. THESES

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M.Sc. in chemistry and English-Hungarian scientific translator

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

*Characterisation of Homo sapiens and Mycobacterium tuberculosis  
dUTPases*

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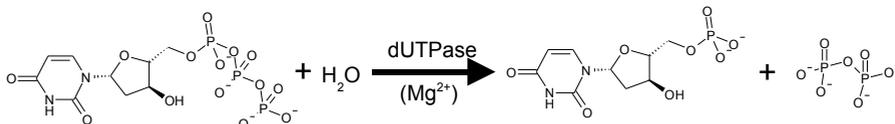
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Sciences

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# 1. INTRODUCTION

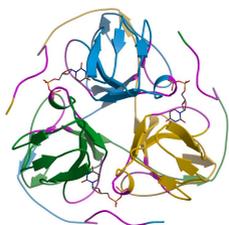
2'-deoxyuridine 5'-triphosphate nucleotidohydrolases (EC 3.6.1.23)

catalyse the hydrolysis of dUTP into dUMP and pyrophosphate:

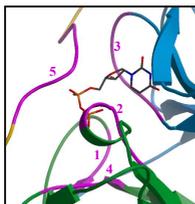


DUTPases are homotrimers with three identical active sites located at the subunit interfaces. There are five highly conserved sequence motifs in each monomer. Each active site is built up by these swapped motifs of the three monomers.

**A**



**B**



**Figure 1. Crystal structure of FIV dUTPase in complex with dUDP (PDB ID: 1F7R). A.** Overall structure. **B.** Close-up of the active site. Motif 1,2 and 4 comes from one monomer, motif 3 from the second one, while motif 5 is provided by the third one.

The dUTPase reaction keeps a low dUTP/dUMP ratio, thus preventing dUTP incorporation into DNA instead of dTTP. It also produces dUMP, the basis of *de novo* dTTP biosynthesis. Enzyme function deficiency leads to thymine-less cell death. Enzymes of *de novo* thymidylate biosynthesis are often targeted by drugs in anticancer and antiviral chemotherapy. These drugs aim to perturb the cellular dUTP/dTTP pools which result in thymine-less cell death preferentially killing cells with actively ongoing DNA synthesis. Targeting dUTPase has therefore been suggested as a promising approach in combination with drugs already in clinical use against thymidylate synthase and dihydrofolate reductase.

The therapeutic potential of inducing thymine-less cell death could be exploited not only in human cancer cells, but also in *Mycobacterium tuberculosis*, where there is no alternative pathway for *de novo* dTTP synthesis, but through dUTPase function.

Despite their importance, the enzymatic properties of neither human nor *M. tub.* dUTPases have been characterised before this study, so our aim was on one hand, to create a quantitative description of human dUTPase enzymatic cycle by revealing the structure-function relations, and on the other

hand to identify species-specific segments of *M. tub.* dUTPase and to develop an activity assay potent for high-throughput screening of potential inhibitors.

## 2. EXPERIMENTAL METHODS

An overexpression system in *E. coli* BL21(DE3) strains was created for the recombinant nuclear isoform of human dUTPase and its different mutant constructs as well as for the recombinant *M. tub.* dUTPase and its mutant construct.

To investigate the thermal stability of human dUTPase, differential scanning microcalorimetry, a useful technique to follow the thermal unfolding of a protein, was applied.

Limited trypsinolysis yields information about the flexible segments of a protein in solution, so it was applied on human dUTPase complexes to investigate any protective effect of the substrate/product..

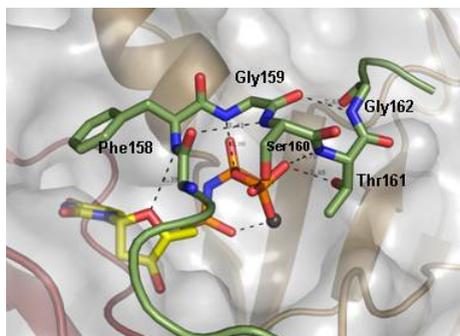
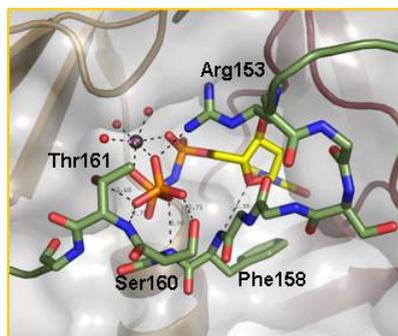
Equilibrium binding studies were performed either by isothermal titration calorimetry or fluorimetry following the heats of binding or the fluorescent signal change of a Trp residue engineered into the dUTPase.

Steady-state kinetic analyses were performed following absorbance in a spectrophotometer, and transient kinetic analyses were performed following fluorescence in a stopped-flow apparatus.

## 3. RESULTS

### 3.1 Structural analysis of human dUTPase

The crystal structure of human dUTPase: $\alpha,\beta$ -imido-dUTP:Mg<sup>2+</sup> complex at a resolution of 2.2 Å was determined in our laboratory. There are novel features in this structure as compared to the previous ones: the full-length C-terminal region is visualized for two monomers, and the structure contains an isosteric substrate analogue in the catalytically competent gauche conformation, as well as the cofactor Mg<sup>2+</sup>. The analysis revealed the followings: There are only two interactions between the C-terminal tail and the dUMP moiety, Phe158 ring stacks over the uracil ring, and the Phe158 amide N H-bonds with the deoxyribose ring (Fig. 2.A). Main chain atoms of residues 159–161 form H-bonds to the  $\beta$ - and  $\gamma$ -phosphates. Arg153 contributes to neutralization of the  $\gamma$ -phosphate negative charge (Fig. 2.B). For the first time, the role of the conserved Ser160 and Thr161 residues, suggested to be of importance, could be assigned. The hydroxyl groups of these residues form H-bonds with the two oxygen atoms of  $\gamma$ -phosphate not involved in chelating Mg<sup>2+</sup> (Fig. 2.B).

**A****B**

**Figure 2. 3D structure of the human dUTPase:α,β-imido-dUTP:Mg<sup>2+</sup> complex. A.** Interactions of the C-terminal tail with the ligand molecule. **B.** Close-up of the interactions formed by Arg153.

The visualisation of the near-attack conformation has also become possible in the two active sites with the full-length C-terminus present, since the distance between the O atom of the nucleophilic attacking water molecule and the α-phosphorus (2.77 and 2.79 Å) is below the H-bond distance (3.32 Å).

### 3.2 Thermal stability

Melting temperature was determined by differential scanning calorimetry, and was found to be 61.7°C, considerably lower than for *E. coli* dUTPase (74.7°C), indicating decreased stability of eukaryotic dUTPases.

### **3.3 Flexibility in solution**

Results of limited trypsinolysis of human dUTPase in its apo-form, in complex with the substrate analogue and in complex with its product, dUMP revealed two main tryptic sites at Arg15 and Arg153 indicating the increased flexibility of the N-terminus, and the C-terminus. The second one, however, was partially protected by both the substrate analogue and the product arguing for the ordering of this segment upon nucleotide binding. Human dUTPase was also found to be approx. 25 times more sensitive against tryptic digestion than the *E. coli* enzyme.

### **3.4 Binding studies by isothermal titration calorimetry**

In order to determine the binding constant of  $\alpha,\beta$ -imido-dUTP, isothermal titration calorimetry was performed. The built-in model was fitted to the binding isotherm, and  $K_d$  was estimated to be  $7.2 \pm 0.2 \mu\text{M}$ .

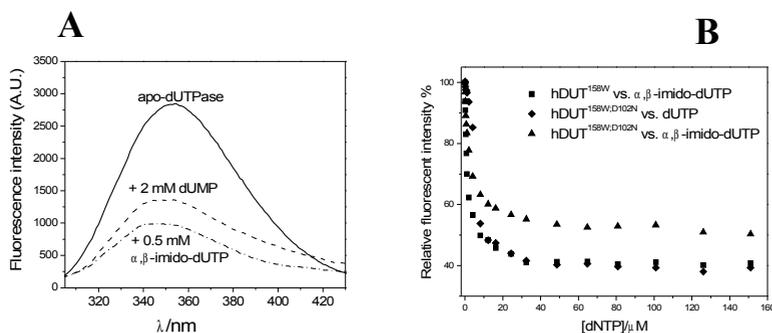
### **3.5 The design of the two Trp mutant constructs**

In order to have a sensitive reporter of the active site conformational changes, Phe158 was mutated to Trp, and equilibrium binding studies were

performed following this intrinsic fluorescent signal. In order to have a saturation curve with the real substrate, Asp102 was mutated to Asn in the above construct to obtain an inactive mutant human dUTPase with a Trp sensor.

### 3.6 Fluorescent equilibrium binding studies

$K_d$  values were found to be  $1.6 \pm 0.2 \mu\text{M}$  for  $\alpha,\beta$ -imido-dUTP and  $26.3 \pm 2.8 \mu\text{M}$  for dUMP (Fig. 3.A).

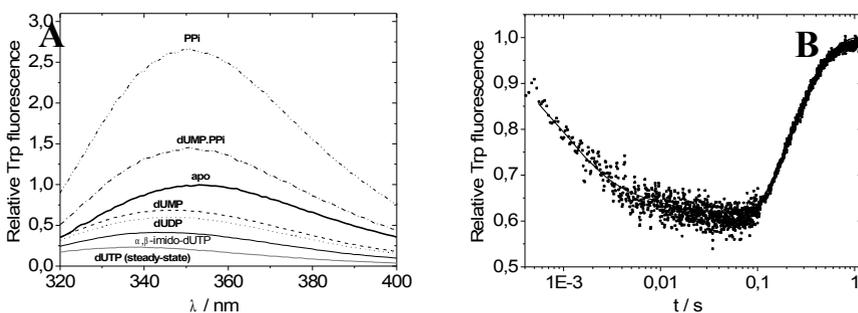


**Figure 3. Equilibrium ligand binding followed by fluorimetry.** **A.** Fluorescent spectra of unliganded dUTPase (straight line), dUTPase: $\alpha,\beta$ -imido-dUTP complex (dashed line) and dUTPase:dUMP complex (dash dotted line) **B.** 3 titration curves are plotted to demonstrate the substrate and its analogue have different binding conformations. Squares represent hDUT<sup>158W</sup> vs  $\alpha,\beta$ -imido-dUTP, diamonds represent hDUT<sup>158W,102N</sup> vs dUTP and triangles represent hDUT<sup>158W,102N</sup> vs  $\alpha,\beta$ -imido-dUTP saturation curves.

Comparison of the saturation curves of the inactive enzyme with the substrate and its analogue reveals the two nucleotides have different binding characteristics (Fig. 3.B).

### 3.7 Transient kinetics

Based on the equilibrium binding studies (cf. the previous section and Fig. 4A), the complex progress curve (Fig. 4B) could be assigned.



**Figure 4. The signal of the intrinsic Trp sensor.** **A.** Fluorescence emission spectra of hDUT<sup>158W</sup> at saturating concentrations of ligands. Data are normalized to the emission peak of the apo enzyme. [hDUT<sup>158W</sup>] = 4 μM, [dUMP] = 500 μM, [dUDP] = 300 μM, [α,β-imido-dUTP] = 100 μM, [dUTP] = 2mM, [PPi] = 5mM. 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. **B.** The graph of a time course upon mixing 7.5 μM hDUT<sup>158W</sup> with 5.25 μM dUTP is shown, prepared for global fitting. The solid line is a global fit to the data points.

The dUTP hydrolysis cycle consists of at least four distinct enzymatic steps (Fig. 5): (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.

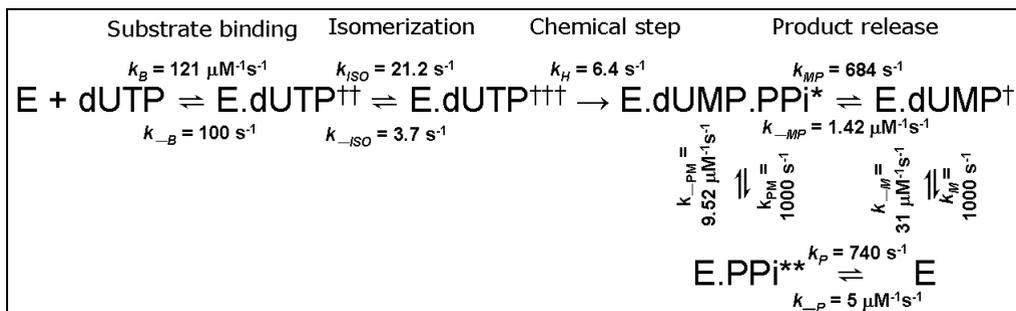


Figure 5. The dUTPase enzymatic cycle.

### 3.8 Structural analysis of *M. tub.* dUTPase

The crystal structure of *M. tub.* dUTPase: $\alpha$ - $\beta$ -imido-dUTP:Mg<sup>2+</sup> complex has been solved at 1.5 Å resolution in our laboratory. The interactions of the residues within the species-specific region with the other parts of the molecule were analysed, and the His145 residue in *M. tub.* dUTPase replacing a highly conserved Phe was also localised. The His145 residue is situated next to the uracil ring of the substrate analogue similarly to the previously observed Phe-uracil stacking in human enzyme. Interactions of

the other species-specific structural element, the C-terminal tetrapeptide, probably facilitate the ordering of Motif 5 upon the active site. 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.

### 3.9 The *M. tub.* dUTPase active site Trp sensor

Based on the structural analysis, the His145 residue was mutated to Trp, and a sensitive sensor was obtained similarly to the human enzyme. *M. tub.* dUTPase binds the substrate analogue,  $\alpha$ - $\beta$ -imido-dUTP with a  $K_d = 0.2 \mu\text{M}$ . This value is significantly different from that of the human enzyme ( $K_d = 1.6 \mu\text{M}$ ), indicating that *M. tub.* dUTPase binds its substrate much stronger than the human enzyme.

### 3.10 Coupled activity assay to detect pyrophosphate formation

The pyrophosphate assay was optimised on the basis of (Webb 1992) and the commercially available EnzCheck Pyrophosphate Assay Kit, in order that the catalytic reaction can be followed continuously (Fig. 6.A). Thus, a



## 4. APPLICATIONS

The previously unavailable structural data of both human and *M. tub.* dUTPases on the crucial C-terminal segment and on the referring part of the active site provide the basis for the design of potentially species-specific inhibitors.

The Trp sensor engineered into the enzymes is an especially sensitive and reliable reporter of the active site conformational changes and enables the detection of active site binders, thus allowing for the determination of the binding constants for the potential active site-binder inhibitors.

The optimised continuous pyrophosphate assay is a robust screening method potent for even large scale screening of potential inhibitors. Since the progress curve of this assay is simply linear, it has an advantage of inhibition as compared to the complex progress curve of Trp fluorescence.

## 5. PUBLICATION LIST

### 5.1 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.

*Kinetic mechanism of human dDUTPase, an essential nucleotide pyrophosphatase enzyme*; J Biol Chem. **282**, 33572-82 (2007):

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 in press:

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.

## 5.2 Publications related to the present thesis

- 1.) **Balázs Varga**, Orsolya Barabás, Júlia Kovári, Judit Tóth, Éva Hunyadi-Gulyás, Éva Klement, Katalin F. Medzihradzsky, Ferenc Tölgyesi, Judit Fidy, and Beáta G. Vértessy: *Active site closure facilitates juxtaposition of reactant atoms for initiation of catalysis by human dUTPase*; FEBS Letters **581**, 4783-4788 (2007).
  
- 2.) Judit Tóth, **Balázs Varga**, Mihály Kovács, András Málnási-Csizmadia, Beáta G. Vértessy: *Kinetic mechanism of human dDUTPase, an essential nucleotide pyrophosphatase enzyme*; J Biol Chem. **282**, 33572-82 (2007).
  
- 3.) **Balázs Varga**, Federica Migliardo, Enikő Takács, Beáta G. Vértessy, 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. Vértessy: *Active site of mycobacterial dUTPase: structural characteristics and a built-in sensor*; BBRC, in press.

### 5.3 Conference proceedings related to the present thesis

- 1.) **Varga, B.**, Barabás, O., Tóth, J., Vértessy, B. G.: Structure-function studies on homotrimeric human dUTPase, SPINE2-Complexes 1st annual conference; Prague, Czech Republic. (2007)
  
- 2.) **Varga, B.**, Due, A., Kuper, J., Wilmanns, M., Vértessy, B. G.: *In vitro* screening of *in silico* identified candidates against two target proteins of *Mycobacterium tuberculosis*, Annual Meeting of the Hungarian Peptide Chemistry Society, Balatonszemes, Hungary. (2007) (in Hungarian)
  
- 3.) **Varga, B.**, Due, A., Kuper, J., Wilmanns, M., Vértessy, B. G.: *In vitro* screening of *in silico* identified candidates against two target proteins of *Mycobacterium tuberculosis*, Alexander von Humboldt workshop; Mátraháza, Hungary. (2007)
  
- 4.) **Varga, B.**, Kovári, J., Barabás, O., Málnási-Csizmadia, A., Hunyadi-Gulyás, É., Medzihradzsky, K. F., Tölgyesi, F., Fidy, J., Vértessy, B. G.: *Structural and kinetic investigations of cooperativity in human dUTPase*, Annual meeting of the Biology Research Center, Hungarian Academy of Sciences; Szeged, Hungary. (2005)

- 5.) **Varga, B.**, Due, A., Kuper, J., Wilmanns, M., Vértessy, B. G.: *In vitro* screening of *in silico* identified candidates against two target proteins of Mycobacterium tuberculosis, EMBO Workshop on Integrated Approaches in Structural Enzymology, EMBL Hamburg, Germany. (2007) (poster presentation)
- 6.) **Varga, B.**, Barabás, O., Hunyadi-Gulyás, É., Medzihradzky, K. F., Tölgyesi, F., Fidy, J., Vértessy, B. G.: Negative cooperativity in human dUTPase homotrimer, EMBO/HHMI CEM2006; Dubrovnik, Croatia. (2006) (poster presentation)
- 7.) **Varga, B.**, Kovári, J., Barabás, O., Takács, E., Málnási-Csizmadia, A., Hunyadi-Gulyás, É., Medzihradzky, K. F., Tölgyesi, F., Fidy, J., Vértessy, B. G.: Cooperativity in human dUTPase homotrimer, FEBS JOURNAL 273, 192-192 (2006). (poster presentation)

## 5.4 Other publications

- 1.) Júlia Kovári, Orsolya Barabás, **Balázs Varga**, Angéla Békési, Ferenc Tölgyesi, Judit Fidy, József Nagy, Beáta G. Vértessy: *Methylene substitution at the  $\alpha$ - $\beta$  bridging position within the phosphate chain of dUDP profoundly perturbs ligand accommodation into the dUTPase active site*; Proteins **71**, 308-19 (2008).