

Thesis of the Ph.D. dissertation entitled:

**„*In vivo* analysis of dUTPase essentiality in *Mycobacterium smegmatis*.
Structural and functional characterization of human and mycobacterial
dUTPase enzymes *in vitro*”**

by

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Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences

2011, Budapest

Background and Objectives

The ubiquitous dUTPase enzyme hydrolyses dUTP to yield dUMP, the precursor for dTTP biosynthesis and pyrophosphate (PP_i). Its action is the only known preventive way to avoid uracil incorporation into DNA, having a major contribution in maintaining the genomic integrity [1]. In all known mycobacterium species, thymidylate biosynthesis necessarily relies on a sole *de novo* biosynthetic pathway which involves the action of dUTPase (encoded by the *dut* gene), in contrast to humans where two *de novo* and one salvage pathway exist. Previous studies have demonstrated the essentiality of the generally occurring dUTPase in *E.coli* and in yeast [2-4]. According to a high throughput genetic study it has been suggested that *M. tuberculosis* requires dUTPase for growth [5]. All known mycobacterial genomes encodes the dUTPase with high similarity, however no detailed information has yet been published about the physiological effect of *dut* knock-outs in mycobacteria. Therefore we may ask the question: What is the physiological effect of *dut* gene knock-outs in *M. smegmatis in vivo*? It is essential? If yes, could serve as a valid drug target molecule? Comparison of the available dUTPase sequences showed that mycobacterial dUTPases possess a unique and mycobacteria-specific insert close to the C-terminus of the polypeptide chain, which is absent from the human enzyme. What might be the physiological role of this mycobacteria-specific loop *in vivo* in *M. smegmatis* and *in vitro* in the enzyme catalysis? It has been shown that the C-terminus of trimeric dUTPases is flexible and is necessary for dUTP hydrolysis [6, 7]. Within this part of the polypeptide chain there is a P-loop like sequence. The amino acids encoded within this P-loop like motif have major contribution in the catalysis of dUTPase, as shown previously in the crystal structures [8, 9]. One of these conserved amino acids is the 158 Phe in the human dUTPase and the 145 His in the mycobacterial enzyme. Both aromatic residues are stacked over the uracil ring in all substrate containing complete dUTPase crystal structures. What could be the role of this aromatic interaction in substrate binding and product release? As known, the dUTPases have an intriguing feature in discriminating the inhibitor dUDP from the substrate dUPNPP, despite the fact that the scissile bond is the α - β phosphate. What is the mechanism by which this discrimination occurs? Does have the P-loop like motif any role in this substrate

discrimination? Previous studies have demonstrated that dUTPase silencing causes embryonic lethality through apoptotic pathway into which the DNA repair mechanism is involved [10]. In the *dut* RNAi *C.elegans* embryos 3 autophagy-related genes were found to be up regulated. Does the CES-2 and ATF-2 protein binds specifically to the promoter sites of the *lgg-1* és *bec-1* autophagy-related genes? To answer this we used EMSA method.

Applied methods:

-Blast analysis: was applied to determine the degree of similarity between the enzymes involved in the thymidylate metabolism including dUTPase of several mycobacterial species.

- “Flexible cassette” method: two step recombination process with application of two plasmid construct (suicide vector and delivery vector); the relevant plasmids were electroporated into *M. smegmatis* cells and thereafter selected for the incoming plasmid.

- Switching strategy: an empty switching vector pSM128 was applied to test gene essentiality; this plasmid contains recombinase and excisionase responsible codons in the sequence.

-Protein engineering: the relevant mutant *M. tuberculosis* and human dUTPases were generated by site directed mutagenesis using the Quick Change kit.

- Protein expression: was performed in *E. coli* BL21pLysS cells. (expressed proteins coding the designed mutation were: WT hDUT, hDUT^{armless}, hDUT^{F158A}, hDUT^{R/K}, hDUT^{ST/AA}, hDUT^{F158W}, mtDUT^{H145W} and mtDUT^{H145A}).

- Protein purification: Ni-NTA affinity chromatography

- Protein concentration: was measured using the Bradford method or by UV absorbance.

- Steady-state kinetics: protons released in the dUTPase reaction were detected by a phenol red indicator assay described in Vertessy *et al.* *BBRC*, 1996.

- Fluorescence and circular dichroism intensity titrations: dissociation constants (K_d) of the mutant and wild type dUTPase-nucleotide complexes were determined using the Jobin Yvon Spex Fluoromax-3 spectrofluorometer and the Jasco 720 spectropolarimeter.

- Rapid-kinetics: fluorescence stopped-flow (Applied Photophysics SX-20) was applied for active site titration and for substrate (dUTP) binding; quench-flow (RQF-3 KinTec Corp., Austin, Texas, USA) experiments were carried out to measure the product release ($^{32}\text{PP}_i$) of the dUTPase catalyzed reaction.
- Crystallization: the mtDUT^{H145W} and mtDUT^{H145A} proteins were co-crystallised with the substrate analog α,β -imido-dUTP, using the hanging drop method.
- Ion-exchange chromatography: was carried out using a Bio-Scale Q2 (Bio-Rad) column in AKTA Purifier (GE Healthcare) chromatography system, to detect the potential dUDP hydrolysis.
- Electrophoretic mobility shift assay (EMSA): the ATF-2 and CES-2 proteins were mixed with *lgg-1*, *bec-1* and *atg-18* autophagy-related gene sequence, and tested for complex formation.

Results (thesis)

1. Comparison of the amino-acid sequences of the thymidylate biosynthesis pathway enzymes from *M. tuberculosis* and *M. smegmatis* revealed that these enzymes exhibit high sequence identity. Blast sequence comparison in other mycobacterial pathogens including *M. leprae*, *M. ulcerans*, and *M. bovis*, showed that all known enzymes in the thymidylate metabolism from these species possess above 84 % identity.
2. Sequence alignment of the C-termini of dUTPases from widely different species (*Echerichia coli*, *Equine infectious anemia virus*, *Vaccinia virus*, *Saccharomyces cerevisiae* és *Homo sapiens*) together with the most relevant mycobacterium species clearly exposes a five amino acid long insert that distinguishes mycobacterial dUTPases from the human and other homologs.
3. The dUTPase *in vivo* knock-outs resulted in lethality in the *M. smegmatis*.
4. We unequivocally confirmed that *dut* gene alone is essential by applying a gene switching strategy.
5. The mycobacteria- specific surface loop is essential for viability in *M. smegmatis*.
6. The deletion of the mycobacteria-specific loop in the *M.tuberculosis* dUTPase did not alter significantly the steady-state activity (1.3-fold decrease in V_{max}) while the dissociation constant (K_d) showed a 11-fold decrease in substrate binding affinity.

7. The phenylalanine aromatic residue (located inside of C-terminal arm within the P-loop like motif) within the active site of dUTPases is conserved.
8. The crystal structure of mtDUT^{H145W} suggests a high degree of conformational conservation of the stacking interaction.
9. The loss of the aromatic interaction does not perturb the overall structure and active site architecture of dUTPase.
10. The loss of the π - π interaction resulted in a decreased steady-state activity (19.3-fold decrease in V_{\max}), while the substrate binding affinity is slightly affected (3-fold decrease in K_d values). After which using the rapid-kinetics methods we have determined that the rate limiting chemical step (hydrolysis) is responsible for decreased activity in the hDUT^{F158A} mutant.
11. Mutations in the P-loop-like sequence disable dUTPase's ability to effectively discriminate between the nucleotide dUDP and dUPNPP (they bind both nucleotides with similar affinity) however the binding of product PP_i was not be detectable.
12. Using the ion-exchange chromatography we showed that the dUDP.BeFx complex is not hydrolyzed by the dUTPase, which further fluorescence intensity measurements confirmed.
13. We have demonstrated *in vitro* that an ATF-2 and CES-2 protein binds to the bec-1 and lgg-1 promoter regions specifically, because when we applied the mutant oligonucleotides no complex formation was detected.

Conclusions

1. We have shown for the first time that the dUTPase enzyme is essential *in vivo* in *M. smegmatis* and the mycobacterium-specific surface loop is also required for viability. Using two comparative genomic approaches we showed that beside the *M. smegmatis* and *M. tuberculosis* other pathogen mycobacterium species share a similar set of enzymes for thymidylate metabolism, with high identity.
2. We have shown that the lack of the mycobacteria-specific surface loop results in minor changes in the enzymatic properties of *M. tuberculosis* dUTPase. This insert induces the formation of a surface loop close to the entrance of the active site as can be seen in the crystal structure. These results and the crystallization dates may lead to the conclusion

that this peculiar motif might serve as a binding surface for a yet unknown protein partner or another ligand. According to this we may hypothesize that this interaction mediates the essential function which we have shown in the *in vivo* experiments.

3. We have shown that the aromatic residue which stacks over the substrate uracil ring within the active site in the dUTPases is conserved. Using rapid-kinetics methods we have proved that in the mutant hDUT^{F158A} the hydrolysis step is responsible for decreased activity. Fluorescence spectroscopy results support our hypothesis, such as the π - π interaction has a major contribution in the transition state stabilization. In conclusion we can propose that this aromatic interaction in the dUTPase promotes the efficacy of catalysis by TS stabilization.

4. We have shown that the disruption of the secondary interaction between the P-loop like motif and the substrate γ -phosphate resulted in a decreased catalytically competent ES-complex formation. Steady-state measurements proved that the hydrolysis occurs in each mutant in extent of the introduced mutation. We may hypothesize that the discrimination between the dUDP and dUPNPP ligands occurs due to the interaction of the P-loop like motif with the γ -phosphate. In addition to that the ion-exchange experiment leads to the conclusion that the entire phosphate ester bond is necessary for an effective hydrolysis to occur on the α -phosphate mediated by the P-loop like sequence.

5. The structural comparison of dUTPase with the relevant *Campylobacter jejuni* bifunctional dUDP/dUTPase and with the *Dictyostelium discoideum* myosin II leads to the conclusion that: *i)* in the case of the bifunctional enzyme the γ -phosphate of the bounded dUTP is coordinated only by water molecules, in contrast to dUTPase where this phosphate is coordinated by the P-loop like sequence; *ii)* the bifunctional dUDP/dUTPase has no P-loop like sequence; *iii)* in case of the type II myosin both phosphates (β and γ) are surrounded by the P-loop, opposite to dUTPase where the P-loop like motif has interactions solely with the γ -phosphate. This difference could be explained by the fact that the P-loop like motif in dUTPase is found in the C-terminus of the protein in a peculiar eclipsed conformation with the substrate.

6. According to this comparison we might propose that the unique role of P-loop like sequence in dUTPase could be gained by functional adaptation, simultaneously with the DNA-repair systems.

7. As a conclusion we can presume that *bec-1* and *lgg-1* autophagy-related gene together with the apoptotic pathway co regulates the early stage of *C.elegans* development. The role of dUTPase in this machinery might be a mediator between stresses and autophagy through its indispensable catalytic activity.

Summary

As a summary I can say that the dUTPase enzyme and its mycobacteria-specific loop motif are essential *in vivo* in the *M. smegmatis*. Peculiarly the deletion of the *M. tuberculosis* dUTPase loop did not diminish the activity. The conserved aromatic interaction has significant contribution in the phosphate ester hydrolysis in dUTPase. The dUDP and dUTP ligands discrimination occurs via the P-loop like sequence and γ -phosphate interaction, as the above presented results demonstrated. Additionally a mediator role might be presumed for the dUTPase in the early stage of *C.elegans* development.

Publications related to the Thesis

Pecsi I*, Leveles I*, Harmat V, Vertessy BG, Toth J. „*Aromatic stacking between nucleobase and enzyme promotes phosphate ester hydrolysis in dUTPase* „ **Nucleic Acids Res.** 2010 Nov 1;38(20):7179-86. Epub 2010 Jul 2. *joint first authors

Erdélyi P, Borsos E, Takács-Vellai K, Kovács T, Kovács AL, Sigmond T, Hargitai B, Pásztor L, Sengupta T, Dengg M, **Pécsi I**, Tóth J, Nilsen H, Vértessy BG, Vellai T. „*Shared developmental roles and transcriptional control of autophagy and apoptosis in Caenorhabditis elegans* ” **J Cell Sci.** 2011 May 1;124(Pt 9):1510-8.

Ildiko Pecsi, Rita Hirmondo, Amanda C.Brown, Anna Lopata, Tanya Parish, Beata G. Vertessy and Judit Toth. „*The dUTPase enzyme is required for mycobacterial viability* ” (under review in the **Journal of Bacteriology**.)

Ildiko Pecsi*, Judit Szabo*, Scott Adams, Istvan Simon, James R. Sellers, Beata G. Vertessy and Judit Toth. „*Nucleotide pyrophosphatase employs a P-loop-like motif to enhance catalytic power and NDP/NTP discrimination*„ (under review in the **PNAS**)

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Oral presentation

Pécsi Ildikó, Hirmondó Rita, Amanda C. Brown, Lopata Anna, Tanya Parish, Vértessy G. Beáta és Tóth Judit. „*A dUTPáz enzim faj specifikus szerkezeti motívumának esszencialitása a Mycobacterium smegmatisban: új célmolekula azonosítása a tuberkulózis gyógyszerterápiában*” IX. Magyar Genetikai Kongresszus és XVI. Sejt- és Fejlődésbiológiai Napok. Siófok, 2011. március 25-27.

Poster presentations (name of the presenting author underlined)

- 1.) I. Pecsí, R.Hirmondo, A. C. Brown, A. Lopata, T. Parish, V. G. Beata and J. Toth P1017, „ *A genus-specific loop in dUTPase is essential for mycobacterial viability* ” 21st ECCMID 27th ICC (European Congress of Clinical Microbiology and Infectious diseases and International Congress of Chemotherapy) Milan, Italy 7-10 May 2011.
- 2.) Ildiko Pecsí, Rita Hirmondo , Amanda C.Brown, Anna Lopata, Tanya Parish, Beata G. Vertessy and Judit Toth. Pos-360: “*Proving the essentiality of dUTPase mediated by its mycobacterium-specific structural motif identifies a novel TB drug target*” Keystone Symposia, Mycobacteria: Physiology, Metabolism and Pathogenesis- Back to the Basics; January 15-20, 2011 Vancouver, Canada.
- 3.) Ildiko Pecsí, Tanya Parish, Amanda C. Brown, Beata G. Vertessy and Judit Toth. Pos-B245: “*Essentiality of dUTPase, a key enzyme in the thymidylate synthesis pathway in mycobacteria*” The EMBO meeting 2010 Barcelona 4-7 September.
- 4.) Ildiko Pecsí, Judit E.Szabo, Beata G.Vertessy, Judit Toth; 2325-Pos: “*The role of P-loop in the enzymatic mechanism of nucleotide pyrophosphatases*” Biophysical Society; February 2010 San Francisco, California.
- 5.) Judit Toth, Ildiko Pecsí, and Beata G. Vertessy. 272.02-Pos “*The Role of γ -phosphate Binding in the Catalytic Mechanism of dUTPase*” Biophys. J. 2008 94: 272-b. San Francisco, California.
- 6.) Judit Toth, Ildiko Pecsí, Scott Adams, Beata G. Vertessy. “*The role of P-loop in the mechanism of α - β phosphate; nucleotide hydrolysis catalyzed by dUTPase*” The International conference on Arginine and Pyrimidines 2008 London.

7.) Pecsi Ildiko, Borsos Eva, Takacs Krisztina, Vellai Tibor, Toth Judit and Vertessy G. Beata. “*Study of the dUTPase enzyme function and mechanism on C.elegans model organism*” Institute of Enzymology, BRC Budapest, Faculty of Science of Eotvos Lorand University, Department of Genetics, Budapest, Hungary 2007.

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