

Structure and function investigation of eukaryotic dUTPases in solution

Ph.D. thesis

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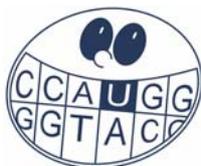
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Research carried out at:

Institute of Enzymology, Hungarian Academy of Sciences

Laboratory of Genome Metabolism and Repair

2009

ACKNOWLEDGEMENT

First of all, I honestly thank my supervisor, **Beáta G. Vértessy D.Sc.**, for giving me the opportunity and inspiring medium to begin research in a new field, for her support and for her advices.

I owe so much to my colleagues for participation of the expression and purification of protein: **Imre Zagyva, Dr. Júlia Kovári, Dr. Orsolya Barabás, Dr. Angéla Békési, Dr. Enikő Takács, Veronika Németh, Villó Muha** and **Ágnes Nagy**.

I wish to thank **Prof. András Perczel** and **Dr. Zoltán Gáspári** for providing me the opportunity to use the NMR and for helping in the interpretation of the spectra.

I also wish to thank **Dr. Éva Hunyadi-Gulyás** for the MS measurements.

I thank **Dr. Csaba Ágoston** and **László Koch** for the ICP-AES measurements.

I wish to thank **Prof. Péter Friedrich** and **Prof. Péter Závodszy**, the former and the present directors of the Institute of Enzymology for allowing me to conduct my Ph.D. research at the institute.

I honestly thank **Prof. Péter Szalay**, my former diploma thesis supervisor for theoretical base and for the special approach.

I thank my colleagues at the Spectroscopic Research Group, Gedeon Richter Plc., for their advices:

Prof. Csaba Szántay Jr., Gábor Balogh, Dr. Zoltán Béni and **Dr. Zoltán Szakács**.

I wish to thank my family, my sons, **Gergő** and **Bulcsú** and my friends for standing by me.

1. Introduction

2'-deoxyuridine 5'-triphosphate nucleotidohydrolases, generally referred to as dUTPases, are essential in bacteria and eukaryotic organisms; moreover, several viruses encode in their genomes their own dUTPase, with nullmutation being lethal. By catalysing the hydrolysis of dUTP into dUMP and pyrophosphate, this enzyme participates in maintaining a sufficiently low cellular dUTP/dUMP ratio. By prohibiting the incorporation of uracil into the DNA, dUTPase preserves the integrity of DNA; its absence results in the so-called thymineless cell death, and its inhibition induces chromosome fragmentation followed by cell death in all cells performing active DNA synthesis (for instance tumor and virus infected cells). Thus research into the dUTPase is a promising new strategy in the development of antiviral and antitumor drug candidates. To that end, the catalytic mechanism of the enzyme and the differences between the different dUTPases that have evolved via different evolutionary pathways should be understood. Our aim was to explore the connection between the catalytic mechanism and structure of dUTPases of different phylogenic states.

Since homotrimeric dUTPases have molecular masses of about 60 kDa, their full three-dimensional structural characterization was not possible on the 500 MHz NMR system available to us. Although X-ray crystallography yields atomic-level structural information, the X-ray structure does not necessarily coincide with the liquid-phase NMR structure, and may therefore not properly represent the molecule's *in vivo* features, as would be needed to analyse the intra-cellular liquid-state processes. Indeed, molecular dynamics play a crucial role in the way proteins work: processes such enzymatic catalysis or substrate binding, always involve the motion of a protein's main- or sidechain. For this reason I chose NMR spectroscopy to investigate the enzyme-substrate interactions and to verify the assumptions regarding the cooperativity of eukaryotic dUTPases, because this method is exceptionally sensitive to molecular interactions and dynamics.

Metal ion co-factors, Mg^{2+} for dUTPases, play a major role in the structure-function relationship. If so magnesium ion, therefore investigation of metal ion effect was thought especially important. Surprisingly, the efficiency of dUTPase catalysis increases owing to its magnesium ion cofactor only by one order of magnitude, whereas other enzymes "working" with different metal ion cofactors, this effect is several orders of magnitude. Therefore I investigated the effect of divalent metal ions on enzyme catalysis and structure.

2. Aims

The present study aimed to achieve the following:

- Investigation of the effect of substrate and substrate analogues binding on the flexible C-terminal segment of dUTPase and verification of the assumed cooperativity of eukaryotic dUTPases by NMR spectroscopy and enzyme kinetic measurements
- Studying the effect of metal ions on enzyme structure stability and on enzyme catalytic mechanism by enzyme kinetic measurements and CD spectroscopy
- Rationalizing the difference between the efficiencies of the pro- and eukaryotic dUTPases

3. Methods

Recombinant dUTPases were produced in BL21 *Escherichia coli*. The protein extracted from digested bacteria cells was purified by ion exchange and gel filtration methods. The purity of dUTPase was controlled on SDS-PAGE (sodium-dodecyl-sulphate). Protein concentration was determined by the Bradford method. Measurement of dUTPase activity was carried out using a spectrophotometric continuous dUTPase enzyme activity assay convenient for kinetic enzyme characterization. Lack of divalent metal ions in buffers were checked by ICP-AES (Inductively Coupled Plasma Atomic Emission Spectroscopy) technique. Any possible decomposition of the protein molecule was controlled by MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time of Flight) mass spectrometry. The role of the flexible C-terminal segment of dUTPases in ligand binding was investigated by multidimensional NMR (Nuclear Magnetic Resonance) spectroscopy. The effect of assorted metal ions on the enzyme structure was investigated by far-UV CD (Circular Dichroism).

4. Scientific results

My investigation of the structure and function of the dUTPase enzymes yielded the following scientific results:

1. In line with our research group's results on limited trypsinolysis (1), I verified that in the case of the *Drosophila* dUTPase, the binding of the product dUMP, or the analogues dUDP and α,β -imido-dUTP, induces a conformational change such that the flexibility of the C-terminus-containing conserved Motif V of the enzyme decreases and folds upon the active region (2).

2. I monitored the effect of the substrates and substrate analogues (dUMP, dUDP, α,β -imido-dUTP) on the conformation of the enzyme's active site by NMR spectroscopy. We titrated the enzyme with each ligand and recorded an ^1H - ^{15}N HSQC spectrum for each titration step. This experiment gave the surprising result that the intensity of the sharp resonances decreased to less than 10 % at only 50 % saturation. This indicates that in all the three titrations partial saturation of the active sites already induces the completion of the conformational shift with regard to folding of the Motif V residues upon the protein globule. To further confirm this hypothesis I recorded a substrate saturation curve. Enzyme kinetics was followed by using spectrophotometric continuous enzyme activity assay. Parametric fitting yielded a Hill-coefficient of 2.8, which indicates strong cooperation (2).

3. The effect of various divalent metal ions was investigated on the catalytic efficiency of prokaryotic *E. coli* and eukaryotic *D. melanogaster* dUTPase. Based on the results of these measurements the investigated divalent metal ions could be divided into three separate groups for both enzymes. Group I contains divalent metal ions which behave similarly to Mg^{2+} in that these ions (Co^{2+} , Mn^{2+} and Ni^{2+}) increased k_{cat} and decreased K_{M} on the same order of magnitude as Mg^{2+} . Group II contains Zn^{2+} , with no detectable effect on the kinetic parameters of dUTPase. Group III contains the alkaline earth metals Ca^{2+} , Ba^{2+} , Sr^{2+} except Mg^{2+} . All three metals decrease the kinetic efficiency $k_{\text{cat}}/K_{\text{M}}$ with respect to its value obtained in the absence of any divalent cation. However, in many respects Ca^{2+} behaves peculiarly. On the one hand, it differs from Sr^{2+} and Ba^{2+} in that it decreases the value of k_{cat} and simultaneously that of K_{M} to the value observed with Mg^{2+} . On the other hand the effect of Ca^{2+} is different for *E. coli* and *D. melanogaster* dUTPase: while the addition of Ca^{2+} to the fruitfly enzyme induces a dramatic tenfold decrease in k_{cat} , the same treatment induces only a 50% decrease of k_{cat} in *E. coli* dUTPase k_{cat} (3).

4. The eukaryotic dUTPases constitute a homotrimer with a central channel wherein the potential accommodation of magnesium and previously selected divalent metal ion was investigated by CD spectroscopy. Here again, three different classes of the metal ions can be established. Group I ions (Mg^{2+} , Co^{2+} , Mn^{2+} and Ni^{2+}) induce a significant enhancement of the negative ellipticity in the CD spectrum of *D. melanogaster* dUTPase. Co^{2+} , Mn^{2+} and Ni^{2+} ions are also similar in their Mg^{2+} -mimicking kinetic effect. Group II contains Sr^{2+} and Ba^{2+} which do not cause a significant effect in the CD signal since they cannot fit into the Mg^{2+} -binding site of the central channel because of their large size and different coordination preference. Group III contains calcium alone which causes a small effect. When similar experiments were conducted with *E. coli* enzyme, no differential CD spectrum could be observed for any of the metal ions (3).

5. Conclusions

In the first part of my work the role of flexible C-terminal of dUTPase in the ligand-binding was investigated by NMR spectroscopy. According to my results, the C-terminal segment loses flexibility upon substrate and also product binding and orders itself to active site of enzyme. This fact is in opposition with results obtained by a Swedish research group with *E. coli* dUTPase (Nord, J., Nyman, P.O., Larsson G. and Drakenberg, T., *Febs Lett* **492**, 228-232 (2001)). This provides new evidence that there is a difference between pro- and eukaryotic enzymatic regulations, since the presence of the folded conformer in the enzyme-substrate complex indicates a cooperation between the subunits.

In the second part of my work the effect of divalent metal ions for enzyme catalysis and structure was investigated. Investigations of this specificity may therefore provide new insights into characteristics of active site structure and catalytic mechanism for dUTPases from different evolutionary branches. Ca^{2+} -inhibition might be structurally given because of the odd number seven as the most preferred coordination number, however, because of more complicated regulation system of eukaryotes (role of concentration changes of calcium ion for example stress, apoptosis) this inhibition effect may get more and more important. The structure of eukaryotic dUTPases is stabilized by a metal ion in the polar central channel which has a potential role in the catalytic mechanism different from its role in the prokaryotic enzymes, since divalent metal ions cannot be accommodated within the inner tight, apolar central channel of prokaryotic dUTPases.

A useful application in X-ray crystallography was also proposed. Accordingly, Co^{2+} and Ni^{2+} ions, similarly to Mn^{2+} , can replace the physiological Mg^{2+} cofactor in a functionally competent manner. The similarity of Mn^{2+} as a cofactor to Mg^{2+} is known and has already been utilized in several other enzyme systems as heavy atoms that facilitate X-ray structure elucidation. Our result about the Mg^{2+} -mimicking d-block divalent metals might thus be helpful in obtaining high resolution structural information of metal-containing dUTPase crystals where Ni^{2+} , Co^{2+} or Mn^{2+} may serve as 'benevolent' heavy atom substitutes.

To summarize, my results indicate that the reason of the difference between the catalytic mechanism of pro- and eukaryotic dUTPases lies within structural alterations. These differences change the communication between subunits, allow the development of cooperativity by eukaryotic dUTPases and may potentiate regulation by calcium ion.

6. Publications

PUBLICATIONS INCLUDED IN THE DISSERTATION

1. **2004** Altered Active Site Flexibility and a Structural Metal-binding Site in Eukaryotic dUTPase: Kinetic Characterisation, Folding, and Crystallographic Studies of the Homotrimeric *Drosophila* Enzyme: J. Kovári, O. Barabás, E. Takács, A. Békési, **Zs. Dubrovay**, V. Pongrácz, I. Zagyva, T. Imre, P. Szabó and B. G. Vértessy *J. Biol. Chem.* 279, 17932-44.

2. **2004** Multidimensional NMR Identifies the Conformational Shift Essential for Catalytic Competence in the 60 kDa *Drosophila melanogaster* dUTPase Trimer: **Zs. Dubrovay**, Z. Gáspári, É. Hunyadi-Gulyás, K. F. Medzihradszky, A. Perczel and B. G. Vértessy, *J. Biol. Chem.* 279 No.17.17945-50.

3. **2009** Divalent metal ion specificity in pro- and eukaryotic dUTPases **Zs. Dubrovay** and B. G. Vértessy (revised version submitted for publication in PlosOne)

ABSTRACTS PUBLISHED IN JOURNAL

2002 dUTPase-dependent preventive DNA repair via exclusion of uracil : J. Kovári, A. Békési, O. Barabás, **Zs. Dubrovay**, I. Zagyva, E. Takács, P. Szabó, T. Imre, A. Erdei, A. Perczel and B. G. Vértessy, *Eur. J. Biochem* 269 PS1 41.

2002 Structural Studies of *Drosophila melanogaster* dUTPase: O. Barabás, **Zs. Dubrovay**, V. Harmat, J. Kovári, E. Takács, I. Zagyva, G. Náray-Szabó, B. G. Vértessy *Acta Cryst.* A58 (supplement), C96.

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ORAL PRESENTATIONS

2002 Form, function, and development in the homotrimeric dUTPase family: O. Barabás, J. Kovári, A. Békési, **Zs. Dubrovay**, I. Zagyva, E. Takács, P. Szabó, A. Perczel., E. Kókay, J. Nagy, **B. G. Vértessy**, International Howard Hughes Research Scholar Meeting, Palm Cove, Australia

2003 A dUTPáz enzimes család szerkezeti biológiája: Katalitikus mechanizmus felderítése szerkezeti alapon (HE-3): **O. Barabás**, **Zs. Dubrovay**, V. Pongrácz, J. Kovári, Z. Gáspári, E. Takács, I. Zagyva, A. Perczel, G. Náray-Szabó, B. G. Vértessy, A Magyar Biokémiai Egyesület Molekuláris Biológiai Szakosztálya 6. munkaértekezlete, Sárospatak