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Nucleocapsid-dUTPase, structure and function of a retroviral protein

Ph. D. Theses

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1. Introduction

The deoxyuridine-triphosphate nucleotidohydrolas (dUTPase) has a unique preventive DNA repair role, controls incorporation of uracil into DNA. The catalyzed reaction is hydrolysis of dUTP into dUMP and pyrophosphate. The dUTPases are homotrimer enzymes with three identical active sites located at the subunit interfaces. The monomers contain five, highly conserved sequence motifs. These motifs create the active centers.

The catalyzed reaction has a dual role within the cell: On one hand, it decreases the cellular dUTP level and keeps the cellular dUTP/dTTP ratio at low level on the other hand, it provides the basis of dTTP biosynthesis by producing dUMP. Absence of dUTPase leads to tyminless cell death. The presence of the enzyme is essential in both pro- and eukaryotes and restricts host range and pathogenicity in both retroviruses and *Herpesvirus*.

In the retroviruses family the lenti- and beta-retroviruses contain the dUTPase enzyme. The beta-retoviral dUTPases are actually bifunctional proteins, because the protein contains two segments, the nucleocapsid (NC) and the dUTPase domains. The question is why these retroviruses need own dUTPase, why they do not use dUTPase of the host cell and why the covalent joining between the NC and dUTPase evolves unlike other retroviruses.

2. Background and aims

Integrity of cellular DNA is endangered by several endogenous and exogenous agents. Incorporation of uracil into DNA may lead to mutation of genome. Cells have several mechanism to retain low uracil content of the genome. The viruses - similarly to the cells - developed some strategies to prevent and repair the uracil mutation in the viral genome.

Genome of the family of herpes-, pox- and retroviruses contain the code of own dUTPase and/or UNG proteins which indicates these viruses are especially sensitive to uracil error in their genome. In family of retroviruses the gene of dUTPase is present in the genome of the beta-retroviruses and the non-primate lentiviruses. These exogenous viruses proliferate in T, cells, B cells and macrophages, thus they are pathogens of several diseases like immunodeficiencies[1]. The exogen beta-retroviruses, like MMTV, M-PMV, JSRV and SRVs are related to some cancer and immunodeficiency diseases.

Why does the genome of these viruses contain the code of dUTPase? The question makes sense, because the code of this enzyme is present in DNA of pro- and eukaryotes. The answer can be that the host cells of these viruses are non-dividing cells and high amount of dUTPase is produced in dividing cells. The lentiviruses proliferate in non-dividing cells. In contrast to oncogene retroviruses need proliferation of their host cell, the lentiviruses can infect the cells arrested in the cell cycle[2]. Role of dUTPase in virus proliferation and pathogenesis was studied in experiments with viruses belong to the lentiviruses infecting non-primate species, like equine (EIAV), caprine (CAEV) and feline (FIV). Turelli and his team created non-dividing cells replication restricted mutant viruses by modifying (via nucleotide insertion and deletion) the dUTPase gene in genome of CAEV[3].

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1 Payne, S.L. and J.H. Elder, *The role of retroviral dUTPases in replication and virulence.* Curr Protein Pept Sci, 2001. **2**(4): p. 381-8.

2 Lewis, P., M. Hensel, and M. Emerman, *Human immunodeficiency virus infection of cells arrested in the cell cycle.* Embo J, 1992. **11**(8): p. 3053-8.

3 Turelli, P., F. Guiguen, J.F. Mornex, R. Vigne, and G. Querat, *dUTPase-minus caprine arthritis-encephalitis virus is attenuated for pathogenesis and accumulates G-to-a substitutions.* J Virol, 1997.**71**(6): p. 4522-30.

Replication of FIV is also restricted at similar circumstances (inactive dUTPase)[4]. In contrast, replication of dUTPase defect CAEV in dividing cells decrease only slightly. This fact suggests, that dUTPase content in proliferating cells can replace the viral dUTPase during the replication and viral life cycle[5]. Experiments with EIAV coded inactive dUTPase gene concluded similar results[6].

Similar experiments was not carried out with beta-retroviruses. Since host cells of these viruses are also non-dividing cells, supposedly the absence of dUTPase can arrest replication of these viruses in similar way.

In my work the goal was characterization of a beta-retroviral, the Mason-Pfizer monkey virus dUTPase. Several data were published about human, *Drosophila melanogaster* or *Eserichia coli* dUTPase, but the retroviral dUTPases are less known enzymes. Among the retroviral dUTPases the beta-retroviral dUTPase has a unique property, namely the covalent joning between two proteins. A nucleocapsid (NC) and a dUTPase domain create a bifunctional protein. First experiments were investigations of catalytic activity and ligand binding of dUTPase domain in presence of NC domain. Protein crystallization and X-ray experiments were planned to determine of 3D structure of the retroviral dUTPase. Role of NC-dUTPase in viral life cycle is not known so the goal was to identify the interacting protein partners of NC-dUTPase.

4 Lerner, D.L., P.C. Wagaman, T.R. Phillips, O. Prospero-Garcia, S.J. Henriksen, H.S. Fox, F.E. Bloom, and J.H. Elder, *Increased mutation frequency of feline immunodeficiency virus lacking functional deoxyuridine-triphosphatase.* Proc Natl Acad Sci U S A, 1995. **92**(16): p. 7480-4.

5 Turelli, P., F. Guiguen, J.F. Mornex, R. Vigne, and G. Querat, *dUTPase-minus caprine arthritis-encephalitis virus is attenuated for pathogenesis and accumulates G-to-a substitutions.* J Virol, 1997. **71**(6): p. 4522-30.

6 Threadgill, D.S., W.K. Steagall, M.T. Flaherty, F.J. Fuller, S.T. Perry, K.E. Rushlow, S.F. Le Grice, and S.L. Payne, *Characterization of equine infectious anemia virus dUTPase: growth properties of a dUTPase-deficient mutant.* J Virol, 1993. **67**(5): p. 2592-600.

3. Methods

The wild type and mutant enzymes were produced in *E. coli* BL21(DE3) cell line.

Flexible regions of NC-dUTPase were identified with VL3H protein disorder predictor (DISPROT) and limited proteolysis.

NC-dUTPase was crystallized to determine the 3D structure of the protein. Structure of apoenzyme, a truncated mutant enzyme and enzyme-substrate complex were determine with X-ray diffraction.

Dynamic protein model was used to determine the location of C-terminal arm of NC-dUTPase. Structure of human dUTPase was the base model.

Small-angle X-ray scattering (SAXS) was applied to measure the conformation changes of dUTPase domain in presence of oligonucleotide.

Far Western dot blot and surface plasmon resonance were used to identify the viral protein interacting partners of NC-dUTPase.

4. Results

4.1. M-PMV contains the dUTPase only in NC-dUTPase form [1].

Western blot analysis of protein content of M-PMV virions was carried out to detect, that only NC-dUTPase fusion protein is present in the virions. An i*n vitro* experiment suggests, that the retroviral protease could not cleave between dUTPase and NC domains.

4.2. Catalytic activity of NC-dUTPase is significantly decreased as compared to other dUTPases [1].

Enzym activity of NC-dUTPase compared to *E. coli*, *D. mel.* or human dUTPases is lower by one order of magnitude. Catalytic activity and ligand binding ability of dUTPase is not affected by the presence of NC domain. The activity increase slightly in presence of oligonucleotide.

4.3. Structure of NC-dUTPase similar to other dUTPases is homotrimer [1].

Dynamic light scattering and analytic gelfiltration were carried out to examine oligomerization of NC-dUTPase. Presence of NC domain do not affect the typical dUTPase homotrimer 3D structure.

4.4. Location of ligand in the active center is similar to human dUTPase [4].

Monomers of M-PMV dUTPase form the usual „jelly-roll" quaternary fold of dUTPase structures. The substrate analogue in a similar way to other dUTPases locates in the active center. The uracil and the dezoxyribose ring interact with ß-hairpin of A monomer of III. motif. The residues of II. and IV. motifs from B monomer act with the phosphate chain. The I. motif in B monomer gives an aspartate residue coordinate the catalytic Mg^{2+} cofactor for the ligand binding. The V. motif of C terminal arm could not be localized on the electron density map due to its flexibility. The C monomer gives the V. motif.

4.5. Conformation of the active center differs from human dUTPase [4].

Conformation of the active center and monomer interaction indicates some interesting difference according to the previously determined dUTPase and dUTPase-ligandum structures. Remarkable difference is primarily in the N- and C-terminal regions. In case of M-PMV NC-dUTPase the N-terminal beta-sheet is missing, since the N-terminal leaves the

protein core to join the NC domain. Because of the missing N-terminal beta-sheet the most important connection between two monomers is lost. Besides, even due to its flexibility the C-terminal arm could not be localized, the electron density map indicates completely different conformation of initial segment of the arm as compared to human enzyme.

4.6. The C-terminal arm is essential for the function of retroviral dUTPase [4].

Mutant enzyme construct with point mutation in the C-terminal arm (Gln220STOP, Arg223Lys) were created. The kinetic constants of these mutant dUTPases decreased $>10^5$-times. This fact suggests the arm is necessary for the function of NC-dUTPase.

4.7. C-terminal arm of NC-dUTPase splices over above the active center of the same monomer[4].

Homology dynamic model was used to clarify the location of the C-terminal arm. The initial model had some restrictions, Arg223 and Phe228 interact the right groups of the ligand, dUTP. Stability of these interactions and flexibility of the arm were tested molecular dynamic simulations in the model, where the C-terminal arm splices over above the active center of the same monomer. After equilibration the molecule was very stable.

4.8. The NC domain retains its Zn^{2+} ion ability [4].

E. coli dUTPase without NC domain was compared to M-PMV NC-dUTPase protein and M-PMV dUTPase without NC domain. Significant fluorescence quenching was observed just in case of NC-dUTPase due to only NC-dUTPase can bind the Zn^{2+} ions.

4.9. The NC domain within the fusion protein in complex with oligonucleotide has more compact conformation than in free form [4].

Based on the structural parameters computed from the SAXS data significant decrease of the hydrated volume of bound NC-dUTPase was observed as well as somewhat smaller values of maximum size and radius of gyration compared to free NC-dUTPase point to compactisation of the protein upon binding of the oligonucleotide. The *ab initio* models of the protein-oligonucleotide complex are similar to those of the free NC-dUTPase but display a somewhat more compact shape in agreement with the analysis of the overall parameters of the samples. To further characterize the structure of NC-dUTPase in solution, rigid body modeling against the experimental data were performed using program BUNCH. The overall shapes were very similar to the corresponding *ab initio* models. Interestingly, the computed scattering of the NC

portion extracted from of the NC-dUTPase model in the absence of the oligonucleotide yields a very good fit to the experimental scattering from free NC in solution. For the oligonucleotide-bound NC, the same procedure yields a somewhat worse fit, indicating that the bound NC in the full-length NC-dUTPase is more compact than the isolated NC in solution complexed with the ligand.

4.10. NC-dUTPase interacts with the other retroviral proteins [3].

The following retroviral proteins were investigated with far western dot blot, integrase (IN), capsid (CA), capsid-nucleocapsid (CA-NC), p12, matrix (MA), nucleocapsid (NC). The IN, CA, CA-NC and NC have given positive results. The interaction between CA-NC and dUTPase was confirmed with surface plasmon resonance.

5. Theses

5.1. NC-dUTPase expressing during the frame shift is the part of the *gag-pro* poliprotein and the viral protease does not cleave the protein between the two domains. Thus, only the dUTPase in covalent joining with NC is expressed in the virion [1].

5.2. Catalytic activity compared to human, *Drosophila melanogaster* and *Escherichia coli* dUTPases is lower by one order of magnitude, but the enzyme activity and ligand binding ability of dUTPase is not affected by the presence of NC domain [1].

5.3. NC-dUTPase binds the ligand in the active center almost in the same way as other dUTPases. C-terminal arm of NC-dUTPase splices over above the active center of the same monomer, contradicting to human and other dUTPases, where the arm interact with the substrate in the active center of neighboring subunit [4].

5.4. The NC protein has Zn^{2+} ion and nucleic acid binding ability. Its functions are retained in the fusion protein. The NC protein in complex with oligonucleotide has more compact conformation than in free form. In the fusion protein ordering of the NC structure is significantly increased [4].

5.5. NC-dUTPase interacts with the following retroviral proteins: integrase, nucleocapsid and capsid-nucleocapsid fusion proteins [3].

6. Application

Experiments with retroviral NC-dUTPase revealed, that the NC domain do not affect function of dUTPase and the NC domain retain – within the fusion protein - its nucleic acid binding ability. The dUTPase in covalent joining with NC could be an improved version of the enzyme. Via its nucleic acid binding ability the NC could help in the localization of dUTPase near the genome, thus dUTPase could hydrolyze dUTP at the most important place.

Presumable connection between different conformation of C-terminal arm of NC-dUTPase compared to human dUTPase and the decreased catalytic activity provides the basis for the design of potential dUTPase inhibitors.

Knowledge of interacting protein partners could provide the basis for design to potential inhibitors. In case of NC-dUTPase interaction with integrase suggests the location of the enzyme in the cell and contributes starting point to development of antiviral drug.

7. Publication list

7.1. Publications related to the present thesis

[1] Barabás O., Rumlová M., Erdei A., **Pongrácz V.**,. Pichová I., Vértessy B.G. *dUTPase and nucleocapsid polypeptids of the Mason-Pfizer Monkey virus form a fusion protein in the virion with homotrimeric organisation and low catalytic efficiency* J Biol Chem. (2003); 278 (40): 38803-12.

[2] Barabás O., **Pongrácz V**., Kovári J., Wilmanns M., Vértessy B.G. *Structural insights into the catalytic mechanism of phosphate ester hydrolysis by dUTPase* J Biol Chem. (2004); 279(41):42907-15

[3] **Németh-Pongrácz, V.**, Snasel, J., Rumlova, M., Pichova, I., Vértessy, G.B. *Interacting Partners of M-PMV nucleocapsid-dUTPase* Nucleosides Nucleotides Nucleic Acids (2006) 25(9):1197-200.

[4] **Németh-Pongrácz, V.**, Barabás, O., Fuxreiter, M., Simon, I., Pichová, I., Rumlová, M., Zábranská, H., Svergun, D., Petoukhov, M., Harmat, V., Klement É., Hunyadi-Gulyás, É., Medzihradszky F.K., Kónya, E., Vértessy, G.B. *Flexible segments modulate co-folding of dUTPase and nucleocapsid proteins* Nucleic Acids Research (2007) 35(2):495-505

[5] Barabás, O., **Németh, V.,** Vértessy, G.B. *Crystallization and preliminary X-ray studies of dUTPase from Mason-Pfizer monkey retrovirus* Acta Crystallograph Sect F Struct Biol Cryst Commun. (2006) 62:399-401

7.2. Conference proceedings

Pongrácz V., Barabás O., Rumlová M., Kónya E., Pichová I., Vértessy B.G. *The bifunctional retroviral nucleocapsid-dUTPase in the virion and in the test tube* (2004) FEBS Forum for Young Scientists, Varsó, Lengyelország

Németh-Pongrácz, V., Barabás, O., Fuxreiter, M., Simon, I., Pichová, I., Rumlová, M., Zábranská, H., Svergun, D., Petoukhov, M., Harmat, V., Klement É., Hunyadi-Gulyás, É., Medzihradszky F.K., Kónya, E., Vértessy, G.B. *Flexible segments modulate co-folding of*

dUTPase and nucleocapsid proteins (2006) Straub napok, Szeged

7.3. Protein structures in Protein Data Bank (PDB)

2D4L **Németh, V.**, Barabás, O., Vértessy, G.B.
Crystal structure of truncated in C-terminal M-PMV dUTPase, 2006 11. 07.

2D4M **Németh, V**., Barabás, O., Vértessy, G.B.
Crystal Structure of apo M-PMV dUTPase, 2006 11. 21.

2D4N **Németh, V.**, Barabás, O., Vértessy, G.B.
*Crystal Structure of M-PMV dUTPase complexed with dUPNPP, substrate analogue,*2006 11. 21.

7.4. Other publications

Kovári J., Barabás O., Takács E., Békési A., Dubrovay Z., **Pongrácz V**., Zagyva I., Imre T., Szabó P., Vértessy B. G. *Altered active site flexibility and a structural metal-binding site in eukaryotic dUTPase: kinetic characterization, folding, and crystallographic studies of the homotrimeric Drosophila enzyme*. J Biol Chem (2004) Apr 23;279(17):17932-44

Békési A., Zagyva I., Hunyadi-Gulyás É., **Pongrácz V**., Kovári J., Nagy Á., Erdei A., Medzihradszky, F.K., Vértessy B.G. *Developmental regulation of dUTPase in Drosophila melanogaster* J Biol Chem (2004) May 21;279(21):22362-70.