Review

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Review

Is dUTPase Enzymatic Activity Truly Essential for Viability?

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Abstract: The study of protein enzymatic activities has always been a significant area of scientific and industrial research. The key stages typically undertaken in the characterization of a given enzyme family include establishing the mechanism of catalysis, measuring kinetic parameters, determining structural organization and the architecture of the catalytic center, and subsequential classification. In this review, we address these classical aspects concerning the dUTPase enzyme family, specifically focusing on spatial structure and catalytic activity, while also providing an overview of certain additional functional properties exhibited by some members of this family. The fact of existence of such extra functions raises questions about the reasons for this functional duality. Based on the information known in the literature and our previous research, in this review we conclude that the enzymatic activity of dUTPases suplements other functions independent of the hydrolysis reaction occurring in the catalytic center. In this context, dUTP acts not just as a substrate, but as a signaling molecule, whose binding facilitates the realization of a distinct, non-enzymatic role of dUTPases.

Keywords: dUTPase; deoxyuridine triphosphate nucleotidohydrolases; dUTP

1. Introduction

All living organisms utilize DNA as the storage and carrier of genetic information. Consequently, cells require systems to protect, edit, repair, and accurately transmit this genetic information. One such system is the DNA repair system, which includes members of the deoxyuridine 5′-triphosphate nucleotidohydrolase (dUTPase) enzyme family. Members of this family are found in all living organisms, as well as in a number of eukaryotic viruses and bacteriophages. These enzymes catalyze the hydrolysis of dUTP into dUMP and a pyrophosphate residue (PPi). As a result, on one hand, the reaction product (dUMP) serves as a substrate for the synthesis of dTTP, a critical component required for DNA replication. On the other hand, this reaction reduces the cellular concentration of dUTP, thereby preventing the erroneous incorporation of uracil residues into newly synthesized DNA. Although the U:A pair is almost indistinguishable from the canonical T:A pair in DNA, the presence of U in newly synthesized DNA triggers the excision repair system. The accumulation of a significant number of uracil residues in DNA can lead to cell death due to the generation of numerous double-strand breaks. Therefore, it is critically important to limit the presence of dUTP in the cell, which might otherwise be erroneously utilized by DNA polymerases during DNA replication.

The dUTPase family encompasses a large number of enzymes that differ in their quaternary structures. These enzymes are classified into three groups: homotrimeric, homodimeric, and monomeric.

The first group, homotrimers, is the most widespread and the best characterized. Homotrimeric dUTPases are found in nearly all living organisms, as well as in several bacteriophages and eukaryotic viruses (such as adenoviruses, poxviruses, and retroviruses). These enzymes possess three

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active sites; each formed at the interface of two adjacent subunits, and are characterized by a beta-sheet folding pattern in the polypeptide chain of the monomer.

Members of the second group are less studied. They have an alpha-helical folding pattern in the main chain of the monomer. Homodimeric dUTPases have been identified in several parasitic organisms, such as *Leishmania major* [1,2], *Trypanosoma cruzi* [3], *Trypanosoma brucei* [4] and *Campylobacter jejuni* [5] as well as in certain staphylococcal bacteriophages [6].

The least numerous group of dUTPases identified to date consists of monomeric enzymes. These are found in certain eukaryotic viruses, specifically in the *Alpha-* and *Gammaherpesvirinae* subfamilies of the *Herpesvirales* [7–12]. Members of this group are characterized by a beta-sheet folding pattern in the main chain and a larger molecular weight compared to members of other enzyme families. It is hypothesized that their origin is associated with the duplication of the gene encoding a homotrimeric dUTPase.

Understanding the structural and functional characteristics of dUTPases provides valuable insights for their potential use as new targets in antimicrobial and possibly anticancer therapies. For example, dUTPase inhibitors may be effective against infectious diseases such as malaria (caused by *Plasmodium falciparum*) and tuberculosis (caused by *Mycobacterium tuberculosis*), as the biosynthesis of dTMP in these pathogens is entirely dependent on dUTPase activity (ссылка). dUTPase is often upregulated in human cancer cells, making it a promising target for anticancer therapy. Studies have shown that the suppression of dUTPase activity in human cells increases their sensitivity to anticancer drugs such as 5-fluorodeoxyuridine, which disrupts dUTP/dTTP levels by inhibiting thymidylate synthase (ссылка). Therefore, targeting multiple enzymes in the thymidylate metabolism pathway could have synergistic effects in combination therapy. Moreover, it has been demonstrated that the replication of certain viruses in non-dividing (terminally differentiated) cells, where cellular dUTPase activity is low or absent, depends on viral dUTPase (ссылка). This likely reflects the importance of viral dUTPase in maintaining genomic integrity and highlights its critical role in the replication of these pathogens.

Despite the critical role performed by dUTPases, viable *E. coli* strains encoding an inactive form of dUTPase have been obtained. At the same time, it is believed that these enzymes perform an additional, as yet unidentified function in the cell. This hypothesis is supported by the observation that dUTPase, along with GroEL, DNA primase, and other *E. coli* proteins, is essential, and knockout of their genes is not feasible [13,14]. Recent studies have also demonstrated that certain dimeric and trimeric dUTPases encoded by bacteriophages regulate the transfer of mobile genetic elements between different strains of *Staphylococcus aureus*, thereby contributing to the dissemination of their pathogenicity genes [15–17].

From the whole pool of available information, we suggest looking out at combination of 4 distinctive features characteristic of this enzyme family: i the global prevalence of the enzyme in living cells; ii high structural diversity; iii mentions of involvement in other processes, iv high substrate specificity to only one not very common molecule. In this context, the question arises whether dUTPase activity is indeed crucial and whether it constitutes the primary function of the enzyme.

2. Structural Organization of dUTPase

As mentioned earlier, all dUTPases are classified into three groups based on their structural organization: homotrimeric, homodimeric, and monomeric. Each group of enzymes is characterized by the presence of specific conserved amino acid motifs and a distinct folding pattern of the polypeptide chain within the monomer.

2.1. Homotrimeric dUTPases

This is the most numerous and well-characterized group of dUTPases to date. Its representatives have been identified in plants, animals, fungi, bacteria, various bacteriophages, and certain eukaryotic viruses, such as adenoviruses, poxviruses, and retroviruses. The three-dimensional structures of a substantial number of trimeric dUTPases encoded by organisms across different

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kingdoms of life have been determined: *Escherichia coli*, *Homo sapience*, several bacteriophages, *Arabidopsis thaliana*, and some of animal viruses [18–23]. As implied by the name of this group, the molecule of homotrimeric dUTPases is composed of three identical polypeptide chains (Figure 1a,c). Each polypeptide is structured as an eight-stranded jelly-roll beta-barrel with one or two alpha-helices. The amino acid sequence of the monomer contains five conserved motifs evenly distributed along the polypeptide chain, which directly participate in the formation of the active site. The spatial structure of each polypeptide chain is such that the amino acid residues of the highly conserved motifs I, II, and IV of one subunit interact with the amino acid residues of motif III from an adjacent subunit. Additional interactions with the dUTP substrate are mediated by the amino acid residues of the conserved motif V, located in the highly flexible C-terminal region of the polypeptide of the third subunit. As a result, each enzyme molecule contains three active sites; each positioned at the interface of two adjacent subunits and formed by the five conserved motifs contributed by all three subunits of the homotrimeric dUTPase.

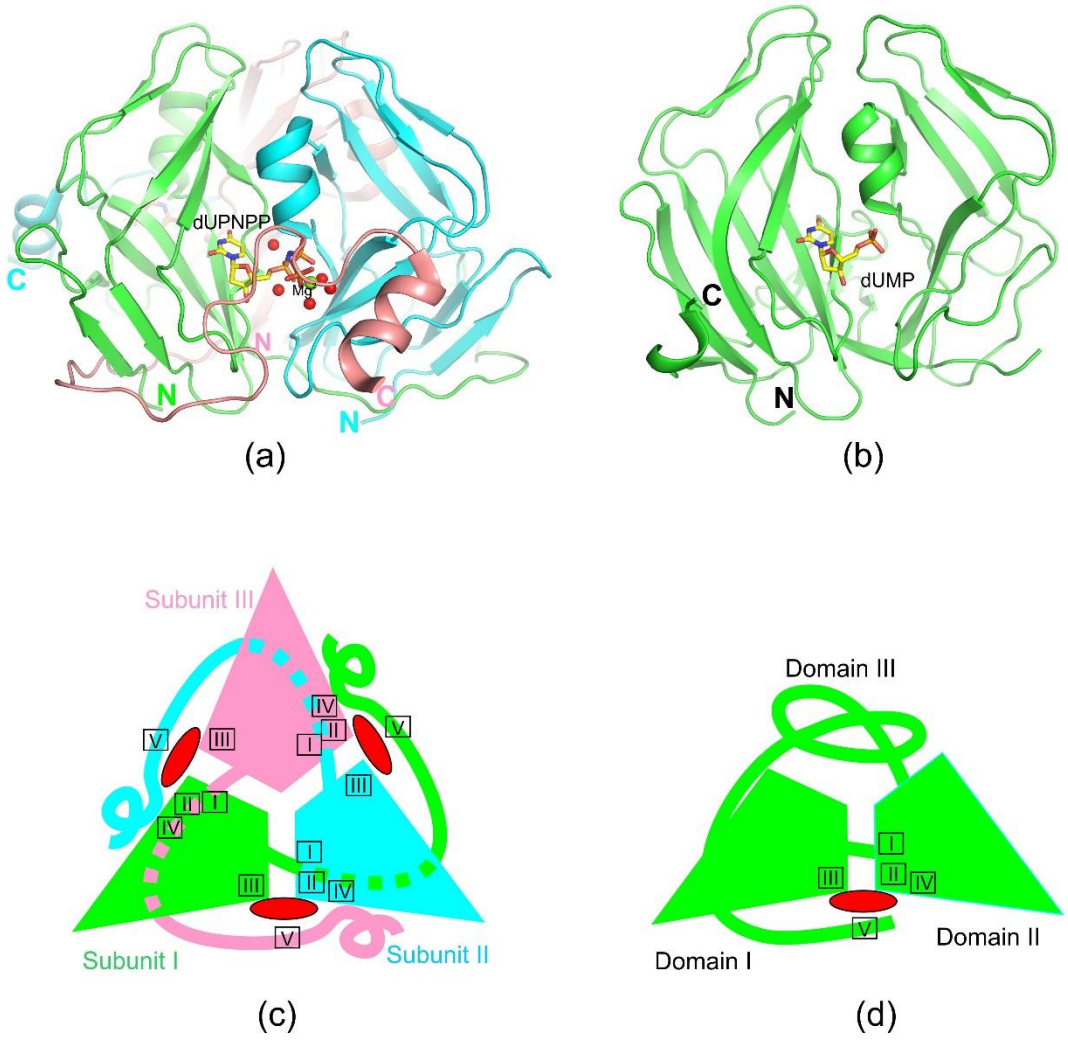

Figure 1. (a) View perpendicular to the 3-fold axis of the Swine enzyme looking onto the active site (pdb id 6LJJ); (b) View looking onto the active site of the monomeric dUTPases Epstein-Barr Virus (pdb id 2BSY) (c) Schematic organization of the trimeric dUTPases and localization of the conserved sequence motifs I–V around the active sites represented by red ellipse. (d) Schematic organization of the monomeric dUTPases.

2.2. Homodimeric dUTPases

This is a small group of dUTPases. They have been identified in several pathogenic microorganisms as well as in certain bacteriophages that infect *Staphylococcus aureus* cells [17,24,25]. In contrast to the previous group, to date, there are only four representatives of homodimeric

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dUTPases that have crystallographic structures available. The three-dimensional structures have been determined for enzymes from pathogenic organisms such as *Trypanosoma cruzi* (PDB: 1OGL), *Leishmania major* (PDB: 2YAY), and *Campylobacter jejuni* (PDB: 2CIC), as well as for the dUTPase of the staphylococcal phage ϕD1 (PDB: 5MYF).

A characteristic feature of dimeric dUTPases is that their monomers are composed of a large number of interwoven α-helices connected by flexible loops. For example, the dUTPases from *L. major* (LjdUTPase) and phage ϕD1 consist exclusively of α-helices (10 and 8 α-helices, respectively), while the dUTPases from *C. jejuni* (CjdUTPase) and *T. cruzi* (TcdUTPase) comprise 11 and 12 α-helices, respectively, along with 2 short β-sheets. Each monomer of a dimeric dUTPase consists of two domains. One domain, the "rigid" domain, participates in the dimerization of subunits and forms the enzyme core. The other, the "flexible" domain, consists of two subdomains formed by the N- and C-terminal amino acid sequences of the monomer, respectively. This domain is positioned at an angle relative to the "rigid" domain, and the entry of the substrate into the enzyme's active site induces a shift in its position by up to 20 Å. Moreover, significant rearrangements of secondary structure elements occur within the "flexible" domain. For instance, structural changes are observed in helix h9 of TcdUTPase, while helices h10 and h12 split into two smaller helices each. Additionally, two new short β-strands are formed. These transformations alter the relative positions of the secondary structures in the "flexible" domain, leading to the formation of a more stable and compact conformation.

The polypeptide chains of homodimeric dUTPases, like those of homotrimeric enzymes, contain five conserved motifs evenly distributed along the entire sequence. However, these motifs share no similarity with those found in trimeric enzymes [1–6]. The amino acid residues within these motifs form the active site and are involved in substrate binding and hydrolysis. Two active sites are symmetrically located at the interface of the dimeric dUTPase molecule, with each substrate-binding pocket situated in a groove formed between the "rigid" and "flexible" domains of a single monomer. As a result, the active site of homodimeric dUTPases is composed of conserved motifs 1, 2, 4, and 5 from one protomer, located in the α-helical regions of the "rigid" and "flexible" domains, and motif 3, positioned in the "clasp" loop of the second subunit.

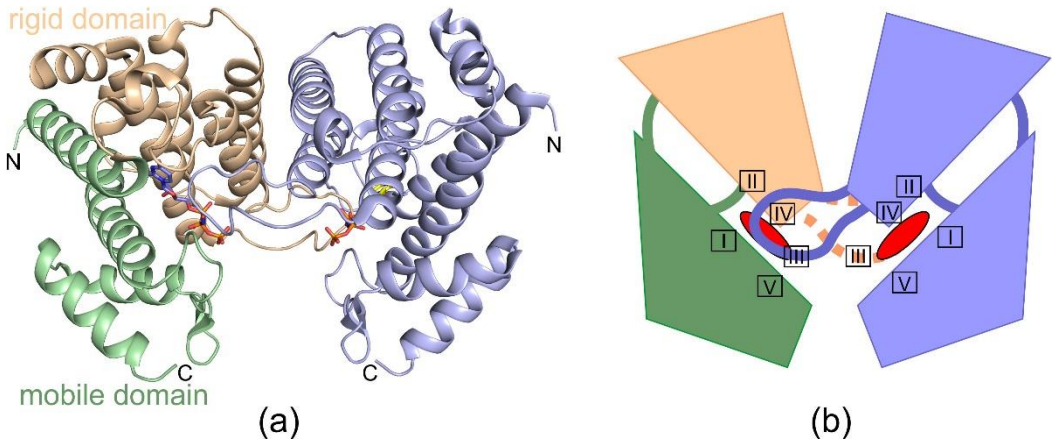

Figure 2. (a) View looking onto the active site of the dimeric from *Campylobacter jejuni* dUTPases (pdb id 2CIC); (b) Schematic organization of the dimeric dUTPases.

It is worth noting that the analysis of amino acid sequences (X-ray structural and bioinformatic analyses) of homodimeric dUTPases, as well as members of the protein families HisE, MazG, and dCTPase of enterophages T2 and T4, suggests that they all belong to a single superfamily of all-α NTP-PPase proteins [24,26,27]. Members of this superfamily are characterized by a shared polypeptide chain folding pattern and similar catalytic properties and functions, specifically in scavenging abnormal NTPs that arise during cellular metabolism. The authors propose that the MazG-like subunit represents a common ancestor in the evolutionary history of this superfamily of enzymes. For example, it is hypothesized that homodimeric dUTPases originated (or evolved)

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through duplication, fusion, and the subsequent loss of one catalytic center in the C-terminal MazG-like domain.

2.3. Monomeric dUTPases

Monomeric dUTPases are found exclusively in viruses of the *Herpesviridae* family, which members infect mammals, birds, and reptiles. Genes encoding monomeric enzymes have been identified in herpes simplex virus (HSV-1) [10], varicella-zoster virus (VZV) [28], Epstein-Barr virus (EBV) [29], and pseudorabies virus (PRV) [30], belonging to the subfamilies *Alphaherpesvirinae* and *Gammaherpesvirinae*.

Currently, structures are available for only two representatives of monomeric dUTPases. The first structure, of the Epstein-Barr virus Dut, was determined in 2005 (PDB: 2BSY) [29], and nearly two decades later, the structure of the pseudorabies virus dUTPase was solved (PDB: 8ZWQ) [30]. These are two-domain proteins, approximately twice the size of a monomer of cellular homotrimeric analogs. However, comparative analyses of amino acid sequences and 3D structures of mono- and trimeric dUTPases reveal significant similarities. For example, the amino acid sequences of monomeric Dut enzymes also contain five conserved motifs characteristic of homotrimeric dUTPases, albeit arranged differently along the polypeptide chain. The shift of motif 3 toward the N-terminus of the protein results in the following sequence: motifs 3, 1, 2, 4, and 5. Similar to homotrimeric dUTPases, enzymes in this group exhibit an almost entirely β-strand structure with a few (1–3) short α-helices.

Despite the low sequence identity between domains I and II of monomeric dUTPases and the subunits of homotrimeric dUTPases, all display a similar jelly-roll beta-barrel fold. Thus, the two domains of a monomeric dUTPase function analogously to the two subunits of homotrimeric Dut enzymes in forming a single active site. The catalytic center is formed by motif 3 from the first domain and motifs 1, 2, and 4 from the second domain, which surround a cavity created by the two domains. Motif 5, located in the mobile C-terminal region of the protein as in homotrimeric dUTPases, likely plays a catalytic role.

Interestingly, PRV Dut exists in a dimeric form in both crystal state and solution [30]. However, unlike homodimeric dUTPases, dimerization of PRV Dut is mediated by a network of hydrogen bonds and hydrophobic interactions between four β-sheets: the short regions P33-V36 and R242-A248 in the N- and C-terminal regions of the protein, respectively. This dimerization stabilizes the protein, which in turn impacts its enzymatic activity. Nevertheless, it cannot be excluded that the oligomeric state of PRV Dut enables additional functional roles during viral replication.

3. Active Site

dUTPase enzymes catalyze the hydrolysis of dUTP, producing dUMP and pyrophosphate as reaction products. This reaction has been most thoroughly described for trimeric enzymes [19,31]. The catalytic process follows an Sn2 mechanism. Substrate hydrolysis is initiated by a linear nucleophilic attack by a water molecule (*Wcat*), which is oriented and activated by a conserved aspartate residue. This attack targets the alpha-phosphate of dUTP, resulting in phosphate ester hydrolysis. The oxygen atom from the attacking water molecule is incorporated into the dUMP molecule rather than the pyrophosphate [31,32]. Specificity in the active site is ensured by precise superposition of the base and sugar moiety, as well as by the proper conformation of the three phosphate groups. This is achieved through multiple water molecules that create an extensive hydrogen bond network and the presence of coordinated magnesium ions. Despite the commonality of the reaction mechanism among mono-, di-, and trimeric dUTPases, the architecture of their active sites varies significantly.

3.1. Homotrimeric dUTPases

The active site architecture of trimeric dUTPases, the most abundant family of these enzymes, is highly conserved across different kingdoms. Despite notable differences in the amino acid sequences

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of various trimeric dUTPases, five conserved motifs have been identified, which are directly involved in forming the catalytic center.

Each trimeric dUTPase, composed of three identical subunits, features three equivalent active sites located at the interfaces between adjacent subunits, forming a symmetric tripartite pattern. Each active site is formed by segments from at least two of the three subunits. Specifically, the catalytic cavity is constructed from motif III of one subunit and motifs I, II, and IV of an adjacent subunit. Motif V, located in the C-terminal region, is often difficult to resolve due to its high mobility. However, when visualized, it is observed to close the catalytic cavity upon substrate binding, isolating the reaction and enhancing catalytic efficiency. The stabilization of motif V's position is mediated by polar interactions between its side-chain residues and the β- and γ-phosphates, as well as hydrophobic interactions between conserved polar residues in the motif and the uracil base.

In most cases, motif V originates from the third subunit, which is not adjacent to the active site. However, exceptions have been reported (e.g., African swine fever virus dUTPase, white spot syndrome virus (wDUT), and *Plasmodium falciparum* dUTPase) [33–35], where the C-terminal region of the protein exhibits a distinct fold. In these cases, motif V contributes to the active site architecture in conjunction with motif III from the same subunit. This unique architectural organization, wherein the formation of the active site depends directly on proper trimer assembly, is unusual among enzymes and underscores the structural and functional sophistication of trimeric dUTPases.

For enzymatic function, dUTPase requires a cofactor, a divalent metal ion. Typically, the active site contains a magnesium ion, which is coordinated via water molecules by conserved residues from motifs I, II, and IV. In the case of the conserved aspartate of motif I, coordination is facilitated through two water molecules. The coordinated magnesium ion interacts with the phosphate groups of the triphosphate, stabilizing the conformation of the intermediate (*gauche*) state of the phosphates and thus enhancing hydrolysis efficiency. Upon the raction completion, magnesium ions may also facilitate the cooperative evacuation of the pyrophosphate product from the active site. This is supported by the fact that certain crystal structures of dUTPases, in their apo-form or in complex with dUMP, do not contain magnesium ions [29,33].

Specificity for uracil and exclusion of purines and thymine are ensured by the β-hairpin formed by conserved residues of motif III [36,37]. In this process, positioning of uracil via hydrogen bonds involves atoms of the main chain rather than side chains, emphasizing that the folding of this region is more critical than the side chain composition. The same hairpin also provides selectivity for deoxyribose, with the conserved tyrosine preventing steric hindrance that would allow ribonucleotide binding. Selectivity for the triphosphate backbone is mediated by conserved residues from motifs I, II, IV and the phosphate conformation induced by the magnesium ion. The spatial structures of dUTPase complexes, where the dUDP is present, show the nucleotide in a 'non-active' trans-state of the phosphate backbone.

Trimeric dUTPases are highly specific enzymes, although some have been predicted (calculated) to bind dATP and dCTP without hydrolytic activity [38].

3.2. Homodimeric dUTPases

Dimeric dUTPases are much less common than trimeric ones, and currently, the structures of active sites have been described for only a few representatives from pathogenic microorganisms [39–41] as well as for several staphylococcal bacteriophages [6]. They share little similarity with trimeric enzymes, including the architecture of their active sites. The level of homology within this group is also low. Nevertheless, similar to trimeric dUTPases, dimeric ones also contain five conserved motifs that organize the active site, though these motifs are distinctly different. The active site cavity is formed by contributions from both subunits. However, the majority of the structural elements are contributed by a single subunit, where motifs II and IV are located within a rigid domain, and motifs I and V are situated in a flexible domain. A long loop, often referred to as the "latch," extends between helices in the inter-subunit space and reaches the neighboring subunit, providing its motif III to contribute to the formation of that subunit's active site.

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The active site of dimeric dUTPases contains either three or two magnesium ions, depending on the ligand present within the active site. Two of these magnesium ions coordinate the catalytic water molecule. In general, amino acid residues from motif I participate in the coordination of the U/C base. Magnesium ion coordination is mediated by residues from motifs II and IV, with motif IV also being responsible for deoxyribose selection. Motif V, located within the flexible domain, together with motif III from the neighboring subunit, coordinates the positioning of the phosphate groups.

Particular attention should be given to the position of A73 in the active site of the staphylococcal bacteriophage ϕDI [6]. Located at the bottom of the substrate-binding pocket, A73 does not actively participate in substrate interaction. However, its substitution with leucine (A73L) creates a steric hindrance that prevents uridine from occupying this site. Consequently, such a point mutation can entirely abrogate substrate binding.

Unlike trimeric dUTPases, where dUDP acts as an inhibitor [32], dimeric enzymes are capable of hydrolyzing both dUTP and dUDP. For this reason, it is suggested that the reaction mechanism may differ from that of trimeric enzymes, requiring more detailed investigation. Dimeric dUTPases are inhibited by dUMP [2]. In addition, these enzymes exhibit reduced nucleotide specificity, demonstrating the ability to hydrolyze dCTP. Such decreased specificity indicates that the active site of dimeric dUTPases has a less rigid organization, allowing the process to occur with greater variability.

A unique representative of this group of enzymes is the pseudorabies virus (PRV), a member of the *Alphaherpesvirinae* subfamily within the swine *Herpesviridae* family. As a dimeric dUTPase, its sequence demonstrates higher homology to the active site sequences of herpesvirus enzymes than to those of described dimeric bacterial enzymes. Alignment reveals six homologous motifs, with conserved residues in five of them contributing to the formation of the active site [30]. Notably, homology to the Epstein-Barr virus (EBV) dUTPase [36] is also observed at the architectural level within the active site, despite the fact that the EBV dUTPase is a monomer. In this context, it would be reasonable to classify this enzyme either within the monomeric group or as a separate group of enzymes specific to the *Herpesviridae* family. Additionally, it is worth noting that disruption of the dimeric structure leads to a significant decrease in enzymatic activity.

Interestingly, the structure of the active sites of the monomers within a single PRV dUTPase molecule differs in topology. One of the monomers exhibits the formation of an additional short α-helix, which undoubtedly contributes to differences in enzymatic activity.

3.3. Monomeric dUTPases

The only currently described spatial structure of a monomeric dUTPase is that of the Epstein-Barr virus (EBV) enzyme [29]. As noted above, its molecule consists of three domains, each positioned analogously to the subunits in a trimeric dUTPase. A single active site is located between domains 1 and 2. It is organized by four out of the five classical motifs described for trimeric dUTPases. Motif V is present in the active site sequence but is not visualized in the described structure due to the high mobility of the C-terminus. All three domains contain nearly the full complement of the five conserved motifs characteristic of trimeric dUTPases, with some exceptions. The motifs forming the single active site exhibit high conservativeness, while those located on other domain surfaces are variable or may be absent. Substrate binding within the active site results in the formation of a similar hydrogen bond network and magnesium ion coordination as observed in trimeric enzymes.

Overall, it can be confidently stated that the reaction mechanism in the active site of the EBV dUTPase is analogous to that of the trimeric enzymes.

4. dUTPase Function

The importance and necessity of dUTPase enzymatic activity for living organisms are, at first glance, indisputable. Indeed, Duts are integral components of the cellular system responsible for maintaining the integrity of genetic material.

The hydrolysis of dUTP to dUMP and pyrophosphate serves two critical functions. First, it maintains a balanced dUTP/dTTP ratio within the cell, thereby preventing the incorporation of uracil

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into newly synthesized DNA. Excessive uracil incorporation during replication can result in numerous irreparable double-strand breaks, ultimately leading to cell death. Second, the reaction product, dUMP, is a key precursor for dTTP synthesis. In pathogenic organisms such as *Plasmodium falciparum* and *Mycobacterium tuberculosis*, which cause malaria and tuberculosis, respectively, dUMP is the sole precursor for dTTP biosynthesis in the cell (ссылочки и во введение).

At the same time, bioinformatic analysis of bacterial and archaeal genomes has revealed that the absence of the *dut* gene is not an exceptionally rare phenomenon among the genomes analyzed [42]. For instance, 14 out of 15 genomes studied within the *Thermotogae* phylum lack *dut* genes. The absence of *dut* genes has also been demonstrated in representatives of the *Planctomycetes*, *Tenericutes*, *Firmicutes*, *Cyanobacteria*, *Spirochaetes*, *Bacteroidetes*, and *Euryarchaeota*. However, it should not be ruled out that the dUTP hydrolysis function in these organisms may be performed by other, as yet unidentified, proteins, such as those from the MazG protein family.

It is worth noting that a significant amount of experimental evidence has accumulated in the literature, highlighting an importance of the mere presence of the dUTPase in the cell rather than its enzymatic function. For example, it has been previously mentioned that the *E. coli* dUTPase belongs to a group of proteins for which gene knockout is not feasible [13,14,43]. However, *E. coli* strains encoding inactive dUTPase do not exhibit significant phenotypic changes. Similar results, highlighting the importance of dUTPase, have also been reported for *Saccharomyces cerevisiae* [44]. In this regard, the idea that dUTPases may possess an additional, as-yet-unknown function that appears to be species- or genus-specific is gaining increasing popularity in the literature. The following experimental data support this hypothesis.

The *M. smegmatis* dUTPase contains a short additional motif VI, consisting of only five amino acid residues, adjacent to motif V in the flexible C-terminal region of the protein. As in the case of *E. coli*, the knockout of the *dut* gene in *M. smegmatis* is lethal for the organism. Although the deletion of this motif does not affect the enzymatic activity of the protein, such mutant form is unable to restore the wild-type phenotype [45]. It is noteworthy that the identified additional motif VI appears to be genus-specific: it has also been identified in the dUTPases of pathogenic organisms such as *M. tuberculosis* and *M. leprae*, where it may also play a role in critical intracellular processes [46,47].

The dUTPase of *Drosophila melanogaster* is notable for existing in two isoforms in actively dividing cells [48,49]. These isoforms, nuclear and cytoplasmic, are generated through alternative splicing and differ by a short nuclear localization signal sequence located at the N-terminal region of the protein. The primary function of the "nuclear" dUTPase is well understood, whereas the function of the "cytoplasmic" isoform remains to be determined. One possible explanation is that this isoform serves an additional role in a signaling pathway.

It is worth noting that *D. melanogaster* dUTPase also contains a species-specific additional sequence of 28 amino acid residues in the C-terminal region of the protein. A similar situation has been observed with rat dUTPase, which has been shown to play a regulatory role in signaling by inhibiting peroxisome proliferator-activated nuclear receptor alpha [50]. The additional sequence in the N-terminal region of the protein is assumed to play an important role in this function. Taken together, these findings support the idea that dUTPases in eukaryotic organisms, like their prokaryotic counterparts, may perform additional functions within cells.

Equally significant and intriguing findings have been obtained from studies of viral dUTPases. Among bacterial viruses, the most extensively studied phenomenon is the induction of staphylococcal pathogenicity island (SaPI) mobilization through the interaction of phage-encoded dUTPase with the Stl repressor [15,16,51]. SaPIs, also known as staphylococcal pathogenicity islands, are genetic elements capable of mobilizing and disseminating virulence genes among *Staphylococcus aureus* strains [52–54]. Under the regulation of Stl, a global repressor encoded by SaPI itself, these genetic elements remain dormant within the host chromosome. However, upon infection of *S. aureus* cells by a bacteriophage, SaPIs are excised, undergo autonomous replication, and are packaged into phage-like particles composed of virion proteins [55,56]. This process results in an exceptionally high frequency of both inter- and intraspecies transfers [57,58]. Both trimeric and dimeric forms of phage-encoded Dut proteins act as antirepressor proteins for specific SaPIs, such as SaPIbov1, SaPIbov5, or

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SaPIov1 [15–17,59]. Comparative analysis of amino acid sequences of homotrimeric Duts from various staphylococcal phages has revealed significant sequence similarity, except for a non-conserved central region designated as motif VI [15]. This motif is absent in some phage-encoded Duts. At the same time, mutant forms of dUTPases lacking motif VI retain enzymatic activity but lose the ability to induce SaPI mobilization. The binding of the Stl repressor by phage dUTPases, while independent of the enzymatic activity of the protein, is competitively influenced by the presence of dUTP in the environment.

Similar findings have been reported for dUTPases of T5-like bacteriophages [18]. It has been demonstrated that the homotrimeric dUTPase of phage T5 performs an additional function during the lytic cycle of the virus, and knockout of its gene leads to dramatic consequences, disrupting phage development within host cells. This additional function, as with other dUTPases, is independent of the enzymatic activity of the protein but is strongly influenced by the presence of a short, additional sequence in the N-terminal region of the protein.

A substantial body of evidence also highlights additional functions of dUTPases encoded by viruses of eukaryotic organisms. For instance, some eukaryotic virus-encoded Duts can modulate the immune response of the infected host. For example, the dUTPase encoded by the pseudorabies virus (PRV) induces lysosomal degradation of the type I interferon receptor, thereby suppressing the alpha-interferon response [60]. Similarly, the dUTPase of MHV-68, while not essential for viral replication, inhibits the expression of the type I interferon signaling pathway [61]. Moreover, the Dut protein from the Kaposi's sarcoma-associated herpesvirus (KSHV) has been reported to suppress the immune response by targeting multiple cytokine receptors. Interestingly, this immunosuppressive function is also independent of the enzymatic activity of the protein [62]. These findings suggest that viral Duts have developed unique immunoregulatory functions.

5. Conclusions

Descriptions of representatives from the class of deoxyuridine triphosphate nucleotidohydrolases traditionally begin with an overview of their enzymatic activity and their capacity to regulate the dUTP/dTTP balance in the organism or host cells. It is invariably noted that a deficiency of this enzyme severely compromises survival, with gene knockouts being lethal. Such outcomes are often bluntly attributed to the accumulation of mutations in actively replicating DNA and the overload of DNA repair systems. However, several observations challenge this plain concept.

First, while the knockout of the *dut* gene is lethal, mutants unable to hydrolyze dUTP are frequently described. Second, some species lack *dut* genes altogether. Third, it has been demonstrated that the frequency of uracil misincorporation during replication, though elevated (approximately six times the standard level), is not high enough to account for total cellular death [42]. Moreover, proteins other than dUTPases can hydrolyze dUTP and may potentially compensate for the absence of the *dut* gene. Conversely, there are proteins with dUTP-binding sites that do not catalyze dUTP hydrolysis.

At the same time, a growing body of research highlights the involvement of dUTPases in processes beyond their enzymatic activity, some of which are unrelated to DNA replication or the dUTP/dUMP balance. Furthermore, numerous examples now exist where the elimination of enzymatic activity—i.e., the ability to hydrolyze dUTP into dUMP and pyrophosphate—does not affect certain parallel properties or functions of dUTPases.

Combining these observations suggests a novel perspective on dUTPases as a group of proteins that are functionally diverse and independently engaged in various vital cellular cascades, while retaining canonical dUTPase activity in either a trimeric or dimeric structural architecture. Consequently, when describing new representatives or functions of dUTPases, it is essential to go beyond enzymatic homology analyses. If one accepts the idea that dUTPase activity coexists with other functions, the high variability in amino acid sequences outside catalytic motifs becomes understandable. Moreover, given that the absence of dUTP hydrolysis itself does not cause critical harm to the organism, unlike *dut* gene knockout, it is plausible that the key factor lies in the ability to bind dUTP. Therefore, studies should focus on mutations that prevent dUTP binding rather than

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merely inhibiting hydrolytic capacity. An example of such approach is the A73L mutation in the dUTPase of bacteriophage ϕDI, which prevents dUTP binding and serves as a model for understanding functional divergence in dUTPases [4].

In light of this concept, the perception of dUTP as a substrate requiring processing undergoes a shift. From being merely a nucleotide—a building block of DNA or an auxiliary supplier of dTTP precursors—it transitions to the status of a molecule used for alternative purposes. A comparable example can be found in the roles of GTP and ATP nucleotides. The classes of GTPases and ATPases encompass a wide variety of proteins that utilize the energy derived from GTP or ATP hydrolysis to perform their primary functions. Structurally, these proteins share domains for binding their respective nucleotides, but their primary functions can differ significantly. In this context, the dUTP molecule may also be viewed as a co-factor whose binding and/or hydrolysis is essential for executing another protein function. Here, the "signal transmission" could be mediated by structural rearrangements upon dUTP binding, such as increased mobility in the C-terminal region of trimeric dUTPases or the transition of two-domain enzymes from an open to a closed form.

Thus, it can be hypothesized that, unlike the energy-providing GTP and ATP, dUTP primarily acts as a signal or trigger in fine-tuned regulatory systems. It is no coincidence that *dut* genes are found across all domains of life, including certain viruses. In viruses—organisms where rapid responses to environmental changes and adaptation of life-sustaining systems are particularly critical—unconventional variants of dUTPase are often observed. These variants, although enzymatically active, frequently exhibit species-specific conservation outside of catalytic motifs. This fact has led researchers to propose viral dUTPase as a promising target for pharmaceutical development.

The notion that the functional diversity of dUTPases arises from a variety of additional functions is supported by cases of discrepancy in the classification of certain enzymes. Traditionally, dUTPases are categorized based on their structural organization, both at the protein level and within the active site. However, there are instances where grouping certain enzymes might better reflect their alternative functions. For example, among staphylococcal viral dUTPases, both trimeric and dimeric representatives exist. Nonetheless, the majority share the ability to interact with the Stl repressor and trigger the mobilization of pathogenicity islands via a common mechanism.

In our studies, dUTPases of T5-like bacteriophages exhibit a distinctive feature: a small loop at the apices of the trimer. This loop demonstrates species-specific conservation within the family, despite variability in its length and sequence. Another example is the dUTPase of PRV. Despite being a dimer with a single active site located between two domains, as is typical for dimeric enzymes, PRV dUTPase might be more appropriately classified as a monomeric enzyme. Furthermore, PRV exhibits phylogenetic affinity to trimeric herpesvirus dUTPases.

At present, the classical classification remains the only viable approach, as identifying additional functions in dUTPases is a challenging task. An even greater challenge lies in pinpointing specific features associated with these additional functions based solely on amino acid sequences. However, as a sufficient body of knowledge accumulates in this field, we may eventually transition to a more refined classification system for this protein family.

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