Kinetic properties and stereospecificity of the monomeric dUTPase from herpes simplex virus type 1

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Abstract Kinetic properties of the monomeric enzyme dUTPase from herpes simplex virus type 1 (HSV) were investigated and compared to those previously determined for homotrimeric dUTPases of bacterial and retroviral origins. The HSV and *Escherichia coli* dUTPases are equally potent as catalysts towards the native substrate dUTP with a k_{cat}/K_M of about 10^7 M^{-1} s^{-1} and a K_M of 0.3 μM. However, the viral enzymes are less specific than the bacterial enzyme. The HSV and *E. coli* dUTPases show the same stereospecificity towards the racemic substrate analogue dUTPαS (2′-deoxyuridine 5′-(α-thio)triphosphate), suggesting that they have identical reaction mechanisms.

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Key words: dUTPase; Nucleotide metabolism; Kinetics; Inhibition; Substrate analogue; Herpes simplex virus

1. Introduction

The ubiquitous enzyme dUTPase, deoxyuridine triphosphate nucleotidohydrolase (EC 3.6.1.23), catalyzes the hydrolysis of dUTP to dUMP and PP_i [1] and plays an important role in DNA synthesis [2]. The enzyme keeps the dUTP/dTTP ratio low, thereby suppressing deleterious misincorporation of uracil into DNA, which could otherwise lead to DNA fragmentation through excision repair and a concomitant increase in rates of mutation and recombination [3]. The hydrolysis of dUTP also provides dUMP, a precursor in the de novo synthesis of dTTP.

dUTPase is encoded by eukaryotes and prokaryotes, and by many viruses. In *Escherichia coli* [4] and *Saccharomyces cerevisiae* [5], the enzyme has been shown to be essential for viability. The retroviral dUTPase is crucial for efficient replication of the virus in non-dividing cells [6–9]. dUTPase from mammalian herpesvirus plays a role when the virus reactivates from latency and dUTPase-deficient mutants show a reduced neuroinvasiveness [10,11]. A dUTPase-dependent drug resistance against 5-fluorodeoxyuridine has been found in a human tumor cell line [12]. These observations indicate that dUTPase may be a potential target for drugs against cancer and viruses.

Most dUTPases, studied so far, are homotrimers. The crystal structures of the trimeric enzymes from *E. coli* [13,14], human cells [15], and lentiviruses [16,17] show how conserved stretches of sequence from different subunits come together forming three identical active sites. Herpesviruses constitute a heterogeneous group; dUTPases encoded by herpesviruses from fish [18,19] appear to conform to the homotrimeric model. The mammalian herpesviruses [20–26] are monomeric and have in general longer polypeptide chains [27,28] with a rearranged order of the conserved sequence motifs [29], implying that the three-dimensional structures of these enzymes are widely different. Therefore, characterization of a dUTPase from a mammalian herpesvirus would be of immediate interest.

We previously cloned the gene encoding dUTPase from a mammalian herpesvirus, HSV, into a pET expression vector [30] for production of the enzyme in *E. coli* and the recombinant enzyme was purified to near homogeneity [28]. Recently, the conditions for gene expression and extraction of the enzyme have been further optimized [31]. Here, we describe the catalytic properties of pure preparations of recombinant HSV dUTPase. The results are discussed and compared to those reported for the homotrimeric dUTPases from *E. coli* [32] and the retroviruses of equine infectious anemia (EIAV) [33] and mouse mammary tumor (MMTV) [34].

2. Materials and methods

2.1. Chemicals

The pH indicators cresol red, nitrazine yellow, and bromocresol purple, and 2′-deoxyuridine, uracil, dUMP, and dUDP were purchased from Sigma. UTP, dUTP, dTTP, dCTP, and dCTPαS were from Pharmacia LKB (Uppsala, Sweden). The substrate analogues dUTPαS (racemic) and α,β-imido-dUTP were prepared as previously described [35,36]. The monodisperse and non-ionic detergent octaethylene glycol mono-*n*-dodecylether ($C_{12}E_8$, with a critical micelle concentration of 7.1×10^{-5} M) was obtained from Nikko Chemicals (Japan). Other chemicals were of the highest available quality.

The nucleotides were purified on a MonoQ HR column (Pharmacia LKB), attached to an FPLC (Pharmacia LKB) with a device for peak integration. The column was equilibrated with 0.01 M HCl in 0.01 KCl and elution performed with a 15-ml gradient of 0.01 M to 0.2 M HCl in 0.01 M KCl. Fractions containing nucleotide were immediately adjusted to pH 7 by adding KOH, and stored at −20°C. Nucleotide concentrations were determined spectrophotometrically. dUTPαS, which is a racemic mixture of two stereoisomers, was digested to completion using HSV dUTPase, 0.5 μM, in 250 μM Bicine, pH 7.5–8.0, 25 μM cresol red, 5 mM $MgCl_2$, 100 mM KCl, and 0.01% (w/v) $C_{12}E_8$. The reaction products, the non-hydrolyzable stereoisomers of dUTPαS and dUMPαS, were purified by the chromatography procedure described above.

2.2. Enzymes

Recombinant HSV dUTPase was purified according to Bergman [31] and stored at −20°C in 20 mM HEPES, pH 7.3, 0.46 M NaCl, 10% (v/v) glycerol, 1 mM dithiothreitol (DTT), and 0.01% (w/v) $C_{12}E_8$. The purified enzyme is stable for years when stored at −20°C. Before the kinetic measurements, the storage buffer was di-

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Abbreviations: dUTPase, deoxyuridine triphosphate nucleotidohydrolase; HSV, herpes simplex virus type 1; EIAV, equine infectious anemia virus; MMTV, mouse mammary tumor virus; dU, 2′-deoxyuridine; α,β-imido-dUTP, 2′-deoxyuridine 5′-(α,β-imido)-triphosphate; dUTPαS, 2′-deoxyuridine 5′-(α-thio)triphosphate; dUMPαS, 2′-deoxyuridine 5′-(α-thio)monophosphate; $C_{12}E_8$, octaethylene glycol mono-*n*-dodecylether

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luted with a solution containing 10 mM $MgCl_2$ and 0.01% (w/v) $C_{12}E_8$ and then concentrated using a dialysis bag (Spectra/por 3) immersed in Na-carboxymethyl cellulose (Aquacide I, Behring Diagnostics). This procedure, repeated until the storage buffer was diluted at least 50 times and the protein concentration was 0.5–2 mg/ml, gives a higher yield of enzyme compared to dialysis or gel filtration. dUTPase from *E. coli* was prepared as described by Hoffmann et al. [37] and stored in 25 mM Bis-Tris, pH 7.0, 0.1 M NaCl at −20°C.

2.3. Kinetic measurements

Measurements of dUTP hydrolysis were performed at 25°C by monitoring the proton production in a weakly buffered solution containing pH indicator using a stopped-flow spectrophotometer (Durrum Model D130, USA; Dionex Model 13000, USA) as previously described [32,33]. The indicator-buffer pairs used were bromocresol purple-MES (pH range 6.1–6.6), nitrazine yellow-MOPS (pH range 6.3–7.2) and cresol red-Bicine (pH range 6.7–9.1). The concentrations of indicator and buffer were 25 μM and 250 μM, respectively, except for bromocresol purple, which was used in a concentration of 50 μM in 125 μM MES buffer. The final concentration of enzyme was 100–200 nM, dUTP 5 or 10 μM, $MgCl_2$ 5 mM, and the ionic strength 0.1 M, obtained by adjustment with KCl. To prevent the dUTPase from precipitating, the detergent $C_{12}E_8$ was added to obtain a final concentration of 0.01% (w/v) during the reaction. The enzyme is stable for several hours in the pH range 6–9. Below pH 6, it rapidly denatures.

2.4. Inhibition

The inhibition of HSV dUTPase by dTTP, dCTP, UTP, dUDP, dUMP, dU, uracil, dUTPαS (the racemate and the non-hydrolyzable stereoisomer), dUMPαS, and α,β-imido-dUTP was determined by including the inhibitor at various concentrations in the assay mixture. The apparent K_M was determined and the inhibition constant, K_i, was evaluated by plotting the apparent K_M versus the inhibitor concentration. All inhibition measurements were performed at pHs close to 8.

2.5. Hydrolysis of other nucleotides

Under the same conditions as used in the stopped-flow measurements, dTTP, dCTP, UTP (1 mM), and dUTPαS (0.15 mM) were incubated at pH 7.5–8.0, overnight, with and without dUTPase (0.5 μM) present. The hydrolysis products were analyzed using ion-exchange chromatography as described in Section 2.1. The enzymic hydrolysis could be run until completion for all substrates except for the racemic dUTPαS, which was hydrolyzed to 50%, corresponding to only one of the two stereoisomers being susceptible to hydrolysis. The non-hydrolyzable isomer was incubated with dUTPase from *E. coli* under equal conditions as above to decide whether the two enzymes have the same stereospecificity.

3. Results and discussion

dUTPases of retroviral [16,17], prokaryotic [13,14], and eukaryotic [15] origin have been investigated by X-ray crystallography and found to be homotrimers. A different structure can be predicted for the dUTPases from the mammalian herpesviruses. These enzymes have longer polypeptide chains, which have probably arisen by gene duplication [29], and are enzymically active as monomers [27,28], findings arguing for a distinctly different molecular organization. Here, we describe a kinetic study of a dUTPase from a mammalian herpes virus, HSV. For the first time, a purified preparation of a dUTPase from this group of viruses has been investigated. The results, presented in Tables 1 and 2, are compared to corresponding data available for homotrimeric dUTPases [32–34].

For the HSV dUTPase (see Table 1), the K_M for dUTP was determined to 0.3 μM, close to the value for the *E. coli* dUTPase (0.2 μM) and lower than the values for the EIAV (1.1 μM) and the MMTV (0.8 μM) enzymes. The k_{cat} for the HSV enzyme was estimated from the V_{max} and the enzyme concentration, determined by UV (280 nm) measurements using the theoretical extinction coefficient ($\varepsilon_{280} = 41\,820\ M^{-1}\ cm^{-1}$), assuming that the enzyme is 100% active. The estimated k_{cat}, 5–15 s^{-1}, is between those of the dUTPases from *E. coli* (6–9 s^{-1}) and EIAV (25 s^{-1}) obtained from single-turnover experiments. The specificity constant, k_{cat}/K_M for dUTP (see Table 2) is, however, of the same magnitude for all three enzymes, while the dUTPase from MMTV, analyzed by a different approach [34], is different.

The HSV dUTPase is highly specific towards dUTP. dTTP, dCTP, UTP and dUTPαS act as competitive inhibitors in the dUTPase reaction. Similar to dUTP, these nucleotides are hydrolyzed to their corresponding monophosphates but at a much slower rate (Table 1). The specificity constants for dCTP and dTTP (approximately $10^3\ M^{-1}\ s^{-1}$, if K_i equals K_M), are 3–4 orders of magnitude lower than for the native substrate dUTP. The enzyme discriminates against dCTP, which has the same molecular shape as dUTP, mainly by poor binding ($K_i = 1$ mM) while the rate of hydrolysis ($k_{cat} \ll 2\ s^{-1}$) is not far from the value for dUTP. The discrimination against dTTP is equally efficient. The binding appears to be slightly stronger (K_i 0.4 mM) than for dCTP but the rate of hydrolysis is about 5 times lower ($k_{cat} < 0.4$

Table 1
Catalytic parameters (K_M (μM) and k_{cat} (s^{-1})) or inhibition constants (K_i (μM)) for viral (HSV, EIAV) and bacterial (*E. coli*) dUTPases towards nucleoside triphosphates and uracil-containing compounds

Substrate	HSV	*E. coli*[a]	EIAV[b]
dUTP			
K_M	0.3	0.2	1.1
k_{cat}	5–15	6–9	25
dUTPαS[c]			
K_M	0.2	0.9	nd
k_{cat}	< 0.2	0.6	nd
dTTP			
K_M[d]	400	> 20000	260
k_{cat}	< 0.4	nd	< 0.5
dCTP			
K_M[d]	1000	4000	3000
k_{cat}	< 2	0.6	< 2
UTP			
K_M[d]	1000	2500	nd
k_{cat}	< 0.2	< 0.3	nd
Inhibitor			
dUMP			
K_i	170	1500	150
dUDP			
K_i	17	15	3.6
dU			
K_i	400	1000	1500
Uracil			
K_i	5000	> 10000	nd
dUPNPP			
K_i	0.9	5[e]	0.6
dUTPαS[f]			
K_i	70	> 50	nd
dUMPαS			
K_i	900	nd	nd

[a]Larsson et al. [32].
[b]Nord et al. [33].
[c]Determined using the racemic mixture.
[d]$K_M \approx K_i$.
[e]Persson et al. [36].
nd, not determined.

Table 2
Catalytic efficiencies (specificity constants, k_{cat}/K_M (M^{-1} s^{-1}))

Substrate	HSV	*E. coli*[a]	EIAV[b]	MMTV[c]
dUTP	4×10^7	4×10^7	2×10^7	2×10^6
dTTP	1000	<100	<2000	2000
dCTP	2000	<100	1000	70
UTP	200	<100	nd	1300

[a]Larsson et al. [32].
[b]Nord et al. [33].
[c]Björnberg and Nyman [34].
nd, not determined.

s^{-1}). For UTP, with a hydroxyl group at the 2′ position of the sugar moiety as the only difference compared to dUTP, the specificity constant is even lower than for dCTP and dTTP. The results of the present study are in agreement with the estimates of specificity constants obtained using a different, discontinuous approach [34]. However, earlier studies using crude extracts [20] and partially purified enzyme preparations [27,38] have given contradicting results, deviating from those obtained here.

Thus, dUTPase from HSV can easily discriminate between dUTP and the other naturally occurring substrates (Table 2). The efficiency is about the same as for the retroviral dUTPase from EIAV. The dUTPase from *E. coli*, on the other hand, has an even higher specificity than the viral enzymes. Its potency towards dUTP is about the same, but the specificity constant for the other nucleotides is decreased by at least one order of magnitude. This difference between the bacterial and the viral enzymes is notable and we would like to suggest that it reflects a difference in the nucleotide metabolism between *E. coli* and the mammalian host systems used by the viruses. In *E. coli*, dUTP is an obligatory intermediate in a compulsory pathway from dCTP to dUMP, the precursor for the de novo synthesis of thymine nucleotides, while in the mammals the major route for synthesis of dUMP is from dCMP. This would imply an elevated flux through dUTP in the bacterium, i.e. a higher enzymic activity, with a concomitant physiological demand for higher enzymic specificity to avoid disturbances in the pools of dTTP and dCTP. The significantly lower product (dUMP) inhibition found for the bacterial enzyme (Table 1) is also consistent with this view.

The second best substrate of HSV dUTPase is dUTPαS. dUTPαS, which has one of the non-bridging oxygens replaced by a sulfur, is racemic. Only one and the same stereoisomer of dUTPαS in complex with Mg^{2+} was found to be hydrolyzed by HSV and *E. coli* dUTPases, suggesting that the enzymes act by the same reaction mechanism. The susceptible stereoisomer of dUTPαS has a very low K_M (0.2 μM), estimated as an inhibition constant as well as by direct measurements in the stopped-flow apparatus, and an apparent k_{cat} (0.2 s^{-1}) about 100 times lower than for dUTP. The non-hydrolyzable stereoisomer has a high K_i (70 μM), which probably reflects an inability to coordinate Mg^{2+} or, more likely, to form a Mg^{2+} complex with high affinity for the active site. The importance of the metal ion has previously been demonstrated by the finding that metal-free dUTP is not hydrolyzed by the *E. coli* enzyme and Mg^{2+} enhances the binding of dUTP by a factor of 100 [32].

dUDP inhibits dUTPase. The K_i is about the same for the HSV and *E. coli* enzymes, while the EIAV dUTPase shows a stronger inhibition. However, dUDP is not hydrolyzed by any of the enzymes, even though the metal ion is present. We believe that the difference in catalytic susceptibility between nucleoside di- and triphosphates is accounted for by a conserved sequence motif [29] resembling the phosphate binding loop found in nucleotide binding proteins [39]. This motif is located in the C-terminal part of dUTPase, invisible in most of the crystal structures [13,14,16,17] and believed to be flexible. Probably, upon binding of a nucleoside triphosphate in complex with a divalent metal ion, this part of the enzyme becomes ordered and a catalytic complex is formed. This idea has recently got support from spectroscopic evidence [40] and is also partly confirmed by the structures for the trimeric human dUTPase in complex with dUMP, dUDP, or dUTP [15], which show an additional ordering of nine amino acid residues of the flexible C-terminus upon binding of nucleotide. However, these complexes do not represent catalytic complexes since the metal ion is missing. Furthermore, the enzyme used is N-terminally truncated compared to native human dUTPase. The finding that the C-terminus of the human dUTPase becomes ordered upon dUDP binding differs from the result previously obtained with the *E. coli* enzyme [14] and the more recently reported structure of EIAV dUTPase [17].

A pronounced requirement for a triphosphate moiety to form a catalytic complex is also consistent with the inhibitory properties of a synthetic triphosphate analogue, α,β-imido-dUTP. This compound was previously reported [36] to be a strong inhibitor of the *E. coli* dUTPase. Table 1 shows that HSV dUTPase is even more strongly inhibited, as is the EIAV enzyme, providing additional support for detailed differences in the structure of the active sites between dUTPases of different origins.

In summary, the monomeric dUTPase from HSV displays kinetic and mechanistic properties comparable to those of trimeric dUTPases indicating a close similarity in the structures of the active sites. Differences in specificity occur between bacterial and viral dUTPases that can be related to differences in nucleotide metabolism. dUTPases of different origins behave differently with respect to inhibitors, a field that will be further exploited in attempts to develop nucleotide analogues for medical applications.

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