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Kinetic properties and inhibition of the dimeric dUTPase-dUDPase from *Campylobacter jejuni*

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Abstract

The enzyme deoxyuridine 5′-triphosphate nucleotidohydrolase (dUTPase) catalyses the hydrolysis of dUTP to dUMP and PPi thus controlling the incorporation of uracil into DNA genomes. In *Campylobacter jejuni* dUTPase exhibits structural properties of dimeric proteins characteristic of protozoa of the Kinetoplastidae family. In the present study we perform a kinetic analysis of *Campylobacter* dUTPase using the continuous spectrophotometric method and show that the enzyme is highly specific for deoxyuridine nucleotides. The Michaelis-Menten constant for dUTP was 0.66 μM while the k_{cat} was 12.3 s^{-1}. dUDP was also efficiently hydrolysed although the specificity constant, k_{cat}/K_m, was five fold lower than for dUTP. The reaction product and the non hydrolysable analogue α,β imido dUDP are potent inhibitors of the enzyme while several analogues of dUMP with substituents at the 3′- and 5′-positions active against trimeric dUTPases, show poor inhibitory activity. Apparent structural and kinetic differences with other eukaryotic dUTPases suggest that the present enzyme might be exploited as a target for new drugs against campylobacteriosis.

Keywords: *duTPase-duDPase, inhibition, kinetics,* Campyrobacter jejuni

Introduction

Deoxyuridine 5-triphosphate nucleotidohydrolase (dUTPase –EC 3.6.1.23) catalyzes the magnesium dependent hydrolysis of dUTP to dUMP and pyrophosphate [1], providing the substrate for methylation of uracil by thymidylate synthase and preventing accidental incorporation of uracil into DNA by DNA-polymerase. Its widespread presence in a variety of organisms, including bacteriophages and certain retroviruses with relatively small genomes, suggests that the dUTPases are vital to DNA replication in all systems [2–4]. dUTPases are present in a variety of different oligomeric forms: monomeric forms are found in herpes virus [5]; homodimers in the Trypanosomatidae and certain bacteria [6,7]; and homotrimers in bacteria and mammals. The homodimeric enzymes lack sequence and structure conservation with the trimeric group of enzymes [8,9]. *Campylobacter jejuni*, is a microaerophilic, Gram-negative, flagellate, spiral bacterium closely related to the gastric pathogen *Helicobacter pylori*. It is the leading cause of bacterial food-borne diarrhoeal disease throughout the world. Not only is gastrointestinal infection with *Campylobacter* the leading cause of bacterial diarrhoeal disease worldwide but also the most common antecedent to the peripheral

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neuropathies Guillain Barre syndrome (GBS) and Miller Fisher syndrome (MFS) [10,11]. *Campylobacter* is a zoonotic pathogen of humans and livestock animals such as cows, pigs, sheep, and farmed poultry are reservoirs for the organism [12].

The complete genome is available [13]. Several genes were found which have homologues only in the eukaryotic domain; these include the deoxyuridine 5′-triphosphate nucleotidohydrolase (dut) gene (Cj1451), which is highly similar to those of *Leishmania major* and *Trypanosoma cruzi* and presents sequence motifs common to the dUTPase-dCTPase of T2 and T4 phages. Conversely, there is no orthologue of the *E. coli* dut gene which belongs to the conserved trimeric family of dUTPases and bears no resemblance with dUTPases of the Trypanosomatidae family. The crystal structure of the *Campylobacter* enzyme has been determined showing that, as other members of this family, dimeric dUTPases have a novel all-α fold and are unrelated to the all-β dUTPases of the majority of organisms including eukaryotes such as humans [14]. A structure-guided analysis of the dimeric dUTPase family revealed its sequence relationship to the phage T4 dCTPase, phosphoribosyl-ATP pyrophosphatase HisE, NTP pyrophosphatase MazG, and several uncharacterized protein families, including the human protein XTP3TPA (RS21-C6) [7]. The unification of these enzymes in one superfamily of all-α NTP pyrophosphatases is proposed, suggesting that dimeric dUTPases evolved from a tetrameric MazG-like ancestor by gene duplication.

In the present report we describe a detailed study of the kinetic properties of the enzyme, assessed by the stopped-flow spectrophotometric method. The bacterial protein exhibits properties typical of dimeric dUTPases lacking the ability to hydrolyze nucleotides other than uridine di and triphosphate.

Materials and methods

Overexpression of recombinant dUTPase of C. jejuni

The coding sequence for *C. jejuni* dUTPase was cloned in pET-11c to give pETCJDUT which was used to transform *E. coli* BL21 (DE3) cells. Transformed cells were cultured at 37°C in LB medium and expression of the enzyme was induced by addition of IPTG (Boehringer) at a final concentration of 1 mM for 3 h. Recombinant dUTPase accounts for 40–50% of soluble protein extract.

For enzyme purification a pellet of dUTPase overexpressing cells was resuspended in a solution consisting of buffer A (Tris-HCl 50 mM, EDTA 2 mM, $MgCl_2$ 2 mM) plus protease inhibitors (leupeptin 0.02 mg/mL, aprotinin 0.05 mg/mL, phenanthroline 10 mM, trypsin inhibitor 0.05 mg/mL, benzamidine 1 mM, PMSF 52μM). The soluble crude extract was obtained by sonication and centrifugation at 11000x*g*. The purification protocol developed consisted in three chromatographic steps (Table I), hydroxyapatite, anion exchange chromatography in DEAE-cellulose, and mono-Q chromatography as previously described for dimeric enzymes [15,16]. This protocol gives 20 mg of protein per liter of culture and the protein is more than 95% free of impurities as judged by SDS-PAGE analysis with Coomassie blue staining of overloaded gels.

Table I. Kinetic parameters for dUTP and dUDP hydrolysis by *Campylobacter jejuni* dUTPase.

Substrate	K_m(μM)	K_i(μM)	k_{cat}(s^{-1})	k_{cat}/K_m(M^{-1} s^{-1})
dUTP	0.66	11.4	12.3	18.6×10^6
dUDP	4.74	50.0	16.6	3.5×10^6

Measurement of dUTPase activity

Nucleotide hydrolysis was monitored by mixing enzyme and substrate with a rapid kinetic accessory (Hi-Tech Scientific) attached to a spectrophotometer (Cary 50) and connected to a computer for data acquisition and storage. Protons, released through the hydrolysis of nucleotides, were neutralised by a pH indicator in weak buffered medium with similar pK_a and monitored spectrophotometrically at the absorbance peak of the basic form of the indicator. The indicator/buffer pair was cresol red/BICINE (pH 8, 573 nm) and the ratio between the indicator and the buffer concentration was 50:2000 (μM). The measurements were performed at 25°C, and the solutions containing 25 mM $MgCl_2$ and 1 mg mL^{-1} BSA were previously degassed. Absorbance changes were kept within 0.1 units. The final enzyme concentration was 0.03 μM. Indicator absorbance changes corresponding to complete hydrolysis of nucleotides were recorded in the computer, and the kinetic parameters (V_{max}, K_{mapp}) were calculated by fitting the data to the integrated Michaelis-Menten Equation 17. Data points in the region of equilibrium and the immediate start were omitted. To obtain the real K_m for substrate hydrolysis and the K_i for product inhibition, the obtained K_{mapps} were reploted versus [S_0]. Units for V_{max} were μmol min^{-1}.

Inhibitors

The preparation of inhibitors was described previously [18].

Table II. Kinetic parameters for dUTP hydrolysis accomplished by monomeric, dimeric and trimeric dUTPases.

Organism (Reference)	Structure	K_m(μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	dUMP: K_{ip} (μM)
C. jejuni	dimer	0.66	12.3	18.6×10^6	11.4
E. coli [20]	trimer	0.2	7.4	3.0×10^7	1500
EIAV[21]	trimer	1.1	25	2.0×10^7	130
MMTV[22]	trimer	0.8	1.5	1.9×10^6	nd
HSV-1[23]	monomer	0.3	12	4.0×10^7	170
L. major[15]	dimer	2.1	49	2.3×10^7	13.1
T.cruzi[9]	dimer	0.5	2.8	5.2×10^6	18.4

Results and discussion

Metal ion requirement

dUTPases depend on the presence of divalent metals in the medium for catalysis. The metal participates in the coordination of the phosphates in the substrate, allowing for a correct orientation and recognition of the substrate in the active site. *C. jejuni* dUTPase was tested for metal requirement measuring activity at different concentrations of magnesium. The enzyme showed a strict requirement for the cation and the V_{max} value increased with increasing concentrations of Mg^{2+} from 0.01 unit mg^{-1} at 0.5 mM to 0.35 units mg^{-1} at 50 mM. No changes in the apparent K_m value were observed even when the Mg^{2+} concentration was raised up to 300 mM.

Hydrolysis of purine and pyrimidine nucleotides

The hydrolysis of different nucleotides by *C. jejuni* dUTPase was studied. The enzyme assay was carried out using as substrates dUTP, dUDP, UTP, dTTP, dCTP, dATP, and dGTP (nucleotides were purchased from Pharmacia except for dUDP and dUTP which were from Jena Bioscience) at a final concentration of 50 μM and 30 nM of enzyme. All nucleotides tested were inefficiently hydrolysed compared to the substrates dUTP and dUDP. Only in the case of dTTP an extremely slow reaction appeared to occur. The determination of the kinetic constants for this pseudo-substrate required very high concentrations of both enzyme and substrate, and the application of the integrated rate equation was unfeasible due to lack of ability to reach a situation of substrate saturation. Kinetic parameters for dUTP and dUDP are summarised in Table I. Values obtained for dUTP hydrolysis are similar to those described for trimeric, dimeric and monomeric dUTPases while the unique capacity for dUDP hydrolysis is shared only with trypanosomatid enzymes (Table II). In addition, the bacterial enzyme is incapable of hydrolysis of nucleotides where the base is not uracil and therefore exhibits features that limit binding of other nucleotides and are responsible for strict substrate specificity.

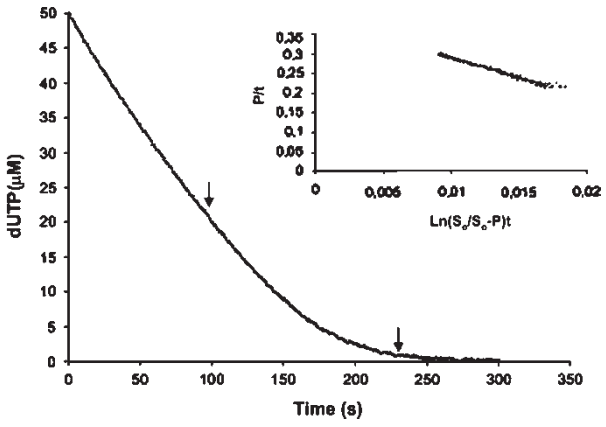


Figure 1. Complete hydrolysis of dUTP by dUTPase under multiple-turnover conditions. The inset shows the linear transformation of the data between the arrows, according to the integrated Michaelis-Menten equation adapted for enzymes with product inhibition, and the corresponding regression line. The reaction was recorded at 573 nm after reacting 30 nm dUTPase and 50 μM dUTP in the presence of 25 mM $MgCl_2$, 2M BICINE, and 50 μM cresol red at pH 8.

Kinetic characterization and inhibition

Recombinant enzyme was purified using a combination of hydroxyapatite, anion exchange chromatography in DEAE-cellulose, and mono-Q chromatography as previously for the dimeric enzymes from the Trypanosomatidae family [15]. Data of the multiple-turnover hydrolysis of dUTP by *C. jejuni* dUTPase was fitted

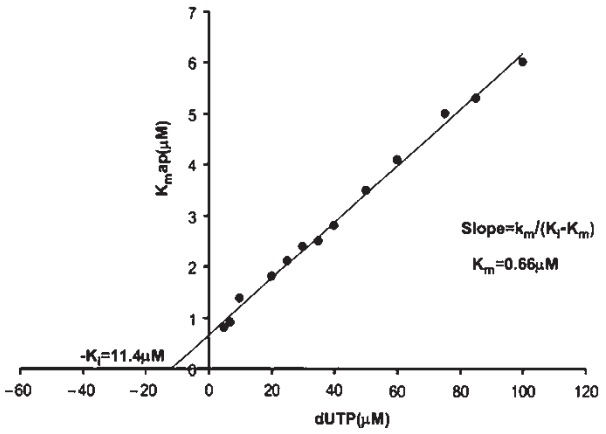


Figure 2. Inhibition by dUMP of dUTP hydrolysis. The K_i value for product (dUMP) inhibition was calculated from the K_{mapp} values obtained at different dUMP concentrations (5 to 100 μM). The K_i for dUMP was 11.4 μM and the real K_m 0.66 μM.

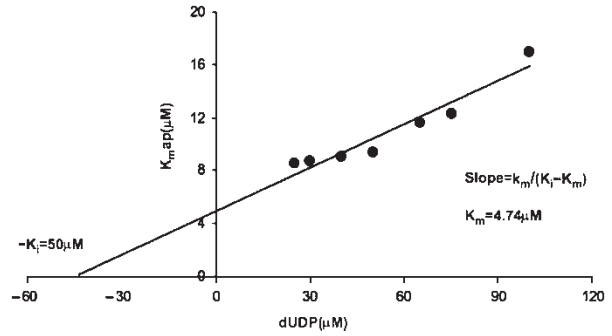


Figure 3. Inhibition by dUMP of dUDP hydrolysis. The K_i value for product (dUMP) inhibition was calculated with the K_{mapp} value obtained at different dUMP concentrations (5 to 100 μM). The K_i for dUMP was 50 μM and the real K_m 4.74 μM.

to the integrated Michaelis-Menten equation as shown in Figure 1, obtaining this way values for K_{mapp} and V_{max}. Both kinetic parameters remained constant with changes in the concentration of buffer/pH indicator. However, while the V_{max} value did not depend on variations in substrate concentration (dUTP), there was an increase in K_{mapp} values with increasing dUTP concentrations, indicating the existence of product inhibition. After replotting the different values of K_{mapp} versus dUTP concentration, the real K_m and the K_{ip} value of product inhibition were calculated (Figure 2).

Using dUTP concentrations between 5 and 100 μM, the real K_m value obtained was 0.66 μM. On the other hand, V_{max} was 0.35 units mg^{-1}, remaining constant at the different dUTP concentrations tested. k_{cat} was estimated to be $12.3^{+}0.5\,s^{-1}$, assuming two active sites per dimer of dUTPase. The specificity constant, k_{cat}/K_m for dUTP was thus $18.6 \times 10^6\ M^{-1}\,s^{-1}$.

Analysis of the hydrolysis data of dUDP as described above for dUTP gave a real K_m value of 4.74 μM, and a product inhibition constant K_{ip} of 50 μM (Figure 3). The k_{cat} value was $16.6\,s^{-1}$ and the specificity constant, K_{cat}/K_m, $3.5 \times 10^6\ s^{-1}M^{-1}$.

The inhibition of *Campylobacter* dUTPase by α-β-imido-dUDP was analysed. For each inhibitor concentration, the apparent K_{mapp} value was obtained using the integrated Michaelis-Menten equation. K_m values are a linear function of the inhibitor concentration in competitive systems and:

$$K_{mapp} = K_m/K_i/(1 - K_m/K_{iproduct})[I] + K^{*}_m(1 + [S_0]/K_{iproduct})/(1 - K_m/K_{iproduct})$$

A replot of K_{mapp} versus [I] has intercepts of $K^{*}_m(1 + [S_0]/K_{iproduct})/(1 - K_m/K_{iproduct})$ (on the K_{mapp} axis) [17]. A K_i value of 1.93 μM was obtained while

Table III. Inhibition constants K_i (μM) for compounds against *Campylobacter jejuni*, *P. falciparum* and human dUTPases.

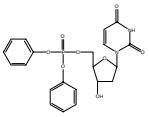
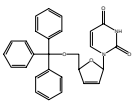
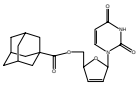
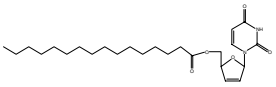
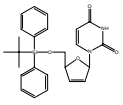
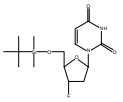
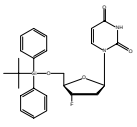
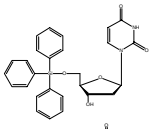
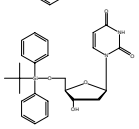
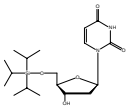
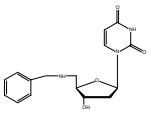
STRUCTURE	K_i(μM) *Human**	K_i(μM) *Campylobacter jejuni*	K_i(μM) *Plasmodium falciparum**
	135	>1 mM	238
	156	>1 mM	1.9
	298	547	57.7
	350	>1 mM	255.7
	>1 mM	>1 mM	1.16
	>1 mM	>1 mM	628
	807	>1 mM	89.39

TABLE III – *continued*

STRUCTURE	K_i(μM) *Human*⋆	K_i(μM) *Campylobacter jejuni*	K_i(μM) *Plasmodium falciparum*⋆
	908	>1 mM	2.8
	805	>1 mM	4.2
	>1 mM	>1 mM	227.1
	>1 mM	>1 mM	110.7

⋆ Values previously reported[18].

no inhibition of the human enzyme was observed at 1 mM inhibitor indicative of a selectivity index greater than 500 for the bacterial enzyme and thus suggesting a basis for specific inhibitor design (Table III).

In addition, a series of analogues of dUMP with substituents at the 3′- and 5′-positions, together with variation in the heteroatom at the 5′-position were tested. Some of these compounds have been previously shown to inhibit efficiently the trimeric *Plasmodium falciparum* dUTPase while they were mostly inactive against the human enzyme [18]. The structural basis for selective inhibition has been determined and is achieved by way of interactions between the trityl group and the side chains of residues Phe46 and Ile117 of the *Plasmodium* enzyme [19]. This emphasises the strong structural and catalytic differences between dimeric and trimeric dUTPases which belong to different protein families and present different inhibition profiles [18]. Further efforts are required for the identification of inhibitors of dimeric enzymes which undergo extensive secondary structure rearrangement with nucleotide binding. In the apo form, the active site is open; upon substrate binding, the enzyme undergoes a large conformational change and closes around the substrate. Thus the nature of the substrate binding site is distinctly different from that in the trimeric (including human) dUTPases [14,6].

In summary, the enzyme of *C. jejuni* has a high capacity of discrimination among the current nucleoside triphosphates present in cells. Kinetic properties and substrate specificity further support that the enzyme is related in function to dimeric enzymes of the Trypanosomatidae family. Likewise, differences in inhibition constants with trimeric enzymes such as human and *Plasmodium* suggest that the design of specific inhibitors of the *Campylobacter* enzyme may be exploited for the identification of new drugs.

Acknowledgements

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