Kinetic properties and inhibition of the dimeric dUTPase-dUDPase from *Leishmania major*

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Abstract

Kinetic properties of the dimeric enzyme dUTPase from *Leishmania major* were studied using a continuous spectrophotometric method. dUTP was the natural substrate and dUMP and PPi the products of the hydrolysis. The trypanosomatid enzyme exhibited a low K_m value for dUTP (2.11 μM), a k_{cat} of 49 s^{-1}, strict Michaelis–Menten kinetics and is a potent catalyst of dUDP hydrolysis, whereas in other dUTPases described, this compound acts as a competitive inhibitor. Discrimination is achieved for the base and sugar moiety showing specificity constants for different dNTPs similar to those of bacterial, viral, and human enzymes. In the alkaline range, the K_m for dUTP increases with the dissociation of ionizable groups showing pK_a values of 8.8, identified as the uracil moiety of dUTP and 10, whereas in the acidic range, K_m is regulated by an enzyme residue exhibiting a pK_a of 7.1. Activity is strongly inhibited by the nucleoside triphosphate analog α-β-imido-dUTP, indicating that the enzyme can bind triphosphate analogs. The existence of specific inhibition and the apparent structural and kinetic differences (reflected in different binding strength of dNTPs) with other eukaryotic dUTPases suggest that the present enzyme might be exploited as a target for new drugs against leishmaniasis.

Keywords: dUTPase; dUDPase; uracil; *Leishmania major*; competitive inhibition; nucleotide metabolism

One of the main steps in the prevention of hazardous incorporation of uracil into DNA by DNA polymerase is the maintenance of a low ratio of dUTP/dTTP (Kornberg and Baker 1991). dUTP pyrophosphatase (dUTPase, EC 3.6.1.23) catalyzes the hydrolysis of dUTP to dUMP, a substrate for thymidylate synthase, and PP_i (Bertani et al. 1961), removing dUTP from the dNTP pool and suppressing DNA fragmentation through excision repair which in turn leads to an increase in the rates of mutation and recombination (Tye et al. 1977).

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Abbreviations: dUTPase, dUTP nucleotidohydrolase; α-β-imido-dUTP, 2′-deoxyuridine 5′-(α-β-imido) triphosphate; dUDPase, dUDP nucleotidohydrolase; dNTP, 2′-deoxynucleoside 5′-triphosphate; DMT-dU, 5′-O-(4–4′-dimethoxytrityl)-2′-deoxyuridine.

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dUTPase is widespread in nature and has been found in a variety of eukaryotic and prokaryotic organisms as well as in many viruses (Bjornberg et al. 1993; Bergman et al. 1994; Climie et al. 1994; McIntosh et al. 1994). The enzyme is essential for viability in *Escherichia coli*, (El-Hajj et al. 1988) and *Saccharomyces cerevisiae* (Gadsden et al. 1993) and is the target of certain cytotoxic drugs in human cancer cells (Zalud et al. 1994). These observations suggest that the activity is essential for DNA replication and cell division.

The three-dimensional structures of dUTPase from *E. coli* (Cedergen-Zeppezauer et al. 1992; Larsson et al. 1996a), feline inmunodeficiency virus (FIV) (Prasad et al. 1996), human (Mol et al. 1996), and equine infectious anemia virus (EIAV) (Dauter et al. 1999) have been determined and reveal a trimeric arrangement of identical subunits, with three active sites, formed by the contribution of residues from each subunit (Larsson et al. 1996; Mol et al. 1996).

Kinetic measurements of the hydrolysis of dUTP by

dUTPase have been improved with time. Traditionally, the activity is measured by separation and quantification of radiolabeled reaction products by TLC (Tye et al. 1977) or by column chromatography (Hoffman 1987), however this method is slow, time-consuming, and discontinuous. The continuous method is based on the fact that hydrolysis of dUTP to dUMP and PP_i is coupled to a pH and pMg-dependent release of protons, so the pH change can be used to follow the reaction. For this purpose, the use of the stopped-flow pH indicator technique has been extended to measure dUTPase reaction kinetic parameters by monitoring complete progress curves. This method has allowed for a detailed kinetic characterization of different dUTPases of distinct organisms like *E. coli* (Larsson et al. 1996b), Herpes simplex virus type I (HSV-I) (Bergman et al. 1995), and EIAV (Nord et al. 1997).

We have reported previously the presence in the parasitic protozoan *Leishmania major* of a cDNA encoding a functional dUTPase, isolated from a cDNA expression library by genetic complementation of dUTPase deficiency in *E. coli*. The gene is of single copy and has an open reading frame (ORF) encoding a protein of 269 amino acid residues, and a theoretical molecular mass of 30.3 kDa. So far, most of the dUTPases described have five amino acid consensus motifs in their sequences, however this parasitic dUTPase lacks these common motifs, and encodes a larger polypeptide (Camacho et al. 1997).

Recently, we have cloned the gene encoding the dUTPase into the expression vector pET-11c, and efficiently overexpressed the protein in *E. coli*. Recombinant dUTPase was purified using a combination of adsorption and anion-exchange chromatography. The enzyme proved to be a dimer in gel filtration and in cross-linking/SDS assays that represents the first time that a dimeric arrangement has been described for a dUTPase. Preliminary measurements using the discontinuous method showed that the enzyme hydrolyzes efficiently dUTP and dUDP into dUMP and PP_i or P_i respectively, and is very sensitive to inhibition by dUMP, but not by PP_i. The activity was highly dependent on the Mg^{2+} concentration (Camacho et al. 2000).

In this report we describe a detailed study of the kinetic properties of the enzyme, assessed by the stopped-flow spectrophotometric method. The results are discussed and compared to those reported for the trimeric dUTPases from *E. coli* (Larsson et al. 1996b), HSV-I (Bergman et al. 1995), the retroviruses of equine infectious anemia (Nord et al. 1997), and mouse mammary tumor virus (MMTV) (Bjornberg and Nyman 1996). Because of the marked differences between this enzyme and the rest of dUTPases described, a detailed study of the kinetic properties will provide valuable information that in the future may aid in the development of specific inhibitors.

Results

Kinetic characterization

Data of the multiple-turnover hydrolysis of dUTP by *L. major* dUTPase could be nicely fitted to the integrated Michaelis–Menten equation for enzymes with appreciable product inhibition, as demonstrated in Figure 1, obtaining these way values of K_{mapp} and V_{max}, as described in Materials and Methods. Both of them remained constant with variations in the concentration of buffer/pH indicator. However, although the V_{max} value was relatively insensitive to variations in substrate concentration (dUTP), there was an increase in K_{mapp} values with increasing dUTP concentrations, reflecting a process 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 can be calculated (Fig. 2). Using dUTP concentrations between 10 and 300 μM, the real K_m value obtained was 2.11 μM and V_{max} was 35.98 ± 1.21 units mg^{-1}. The hydrolysis

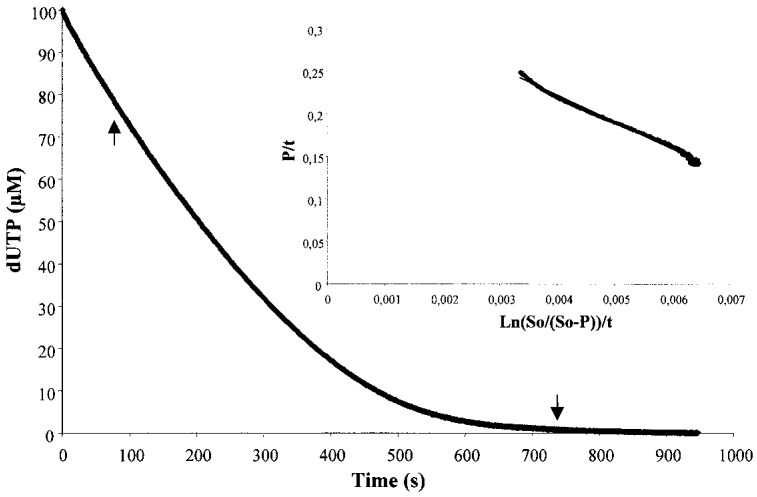

Fig. 1. Complete hydrolysis of dUTP by dUTPase under multiple-turnover conditions. The inset shows the linear transformation of the data between the arrows, according to the integrated Michaelis–Menten equation adapted for enzymes with product inhibition as described in Materials and Methods, and the corresponding regression line. The reaction was recorded at 573 nm after reacting 30 nm dUTPase and 25 μM dUTP in the presence of 25 mM $MgCl_2$, 2500 μM BICINE, and 50 μM cresol red at pH 8.

product, dUMP, caused competitive inhibition with a K_{ip} value of 13.05 μM. This low K_{ip} value was confirmed by the addition of different concentrations of dUMP (5–20 μM) at t = 0. Thus, different values of K_{mapp} were obtained and used for the analysis of competitive inhibition as described in Materials and Methods for α-β-imido-dUTP. The K_{ip} value obtained using this method was 12.35 μM (Fig. 3). However, the other product of the reaction, PP_i, did not inhibit the enzyme from *L.* (data not shown). Single turnover measurements gave a k_{cat} value of 49 s^{-1}. The specificity constant, k_{cat}/K_m for dUTP was thus 2.3×10^6 $s^{-1}M^{-1}$.

dUDP appeared to be an efficient substrate for dUTPase with a V_{max} of 99.38 ± 2.58 units mg^{-1}. Analysis of the hydrolysis data of dUDP as described above for dUTP gave a real K_m value of 62.70 μM, and a product inhibition constant (K_{ip}) of 130.75 μM. The k_{cat} for dUDP hydrolysis was calculated from the V_{max} and the enzyme concentration, estimated from the extinction coefficient at 280 nm. Assuming that the enzyme was 100% active and that there are two active sites per dimer, the estimated k_{cat} was 65 s^{-1} and the specificity constant, k_{cat}/K_m, was 1×10^6 $s^{-1}M^{-1}$.

PP_i release

The release of pyrophosphate during the hydrolysis of dUTP was analyzed as described in Materials and Methods. A blank reaction, containing exactly the same volume as the sample to analyze and a control reaction, which was a standard sample of a known PP_i concentration (16 μM), were analyzed. The control was used to calculate the apparent extinction coefficient of NADH. Samples with dUTP incubated 5 min in the presence of the enzyme were measured. The calculated PP_i concentration was 24.71 ± 1.61 μM, which is equivalent to the initial dUTP concentration included in the reaction mixture suggesting that PP_i and dUMP are the sole products of the reaction.

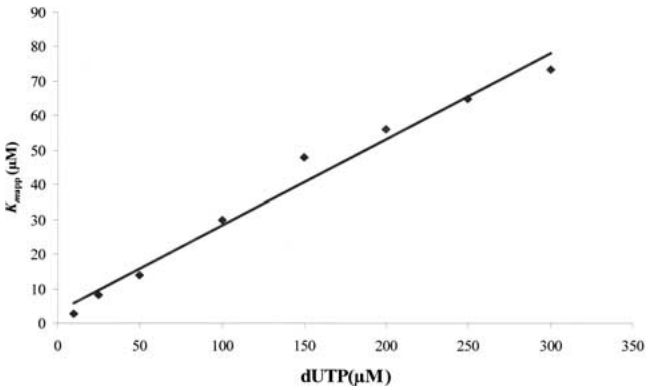

Fig. 2. Replot of K_{mapp} vs. $[S_0]$. Replot of the different slope values (K_{mapp}) from the linear plots of the inset of Figure 1, at different substrate concentrations from 10 to 300 μM, vs. $[S]_0$. Following the integrated Michaelis–Menten equation for enzymes with product inhibition, the K_{ip} value and the real K_m could be obtained from this new linear replot as described in Materials and Methods.

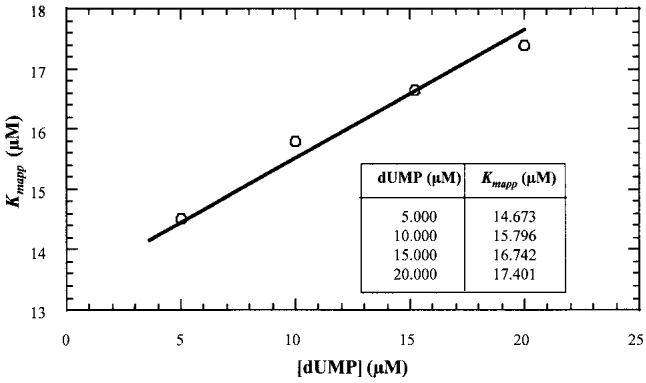

Fig. 3. Inhibition by dUMP. The K_i value for product (dUMP) inhibition was calculated with the K_{mapp} values obtained at different dUMP concentrations (5, 10, 15, and 20 μM using the formula shown in Materials and Methods for competitive inhibitors. The value obtained was 12.35 μM. The reactions were carried out using 50 μM dUTP and 30 nM dUTPase at pH 8 and 25°C.

Effects of pH on V_{max} *and* K_m

The kinetic parameters for the hydrolysis of dUTP, V_{max} and K_{mapp}, were determined in the pH interval 5.5–10, using different pairs of buffer/pH indicator. The V_{max} value was relatively constant at pH 5.5–7 and 8.5–10, however there was a five-fold increase between pH 7–8.5 (Fig. 4a). This increase in activity suggested the existence of a residue with pK_a values in that range that may be important for catalysis. The corresponding values of K_{mapp} at different pH are shown in Figure 4b and imply that substrate binding is affected by at least three proton dissociations, according to the equation: $K_{mapp} = K_m([H^+]/K_1 + 1 + K_2/[H^+]\{1 + K_3/[H^+]\})$. The best fit of the data to this equation was obtained for $K_m = 2.35$ μM, revealing three pK_a values for residues involved in substrate binding: $pK_1 = 7.1$, $pK_2 = 8.8$, and $pK_3 = 10$. Titration of dUTP has been described to give a pK_a of 8.9 which corresponds to the ionization of the uracil moiety (Dawson et al. 1986; Larsson et al. 1996a,b).

Effects of temperature on V_{max} *and* K_m

Analysis of V_{max} and K_m values at different temperatures from 4°C to 60°C allowed us to conclude that the K_m value is apparently insensitive to temperature variations. However, clear changes in V_{max} occurred. From 4°C to 50°C there was an increase in activity, reaching a maximum at 50°C. At higher temperatures (≈60°C), dUTPase activity decreased very quickly, and in a short time was no longer detectable due to denaturation. In the interval from 4°C to 55°C, where denaturation is negligible, the increase of ac-

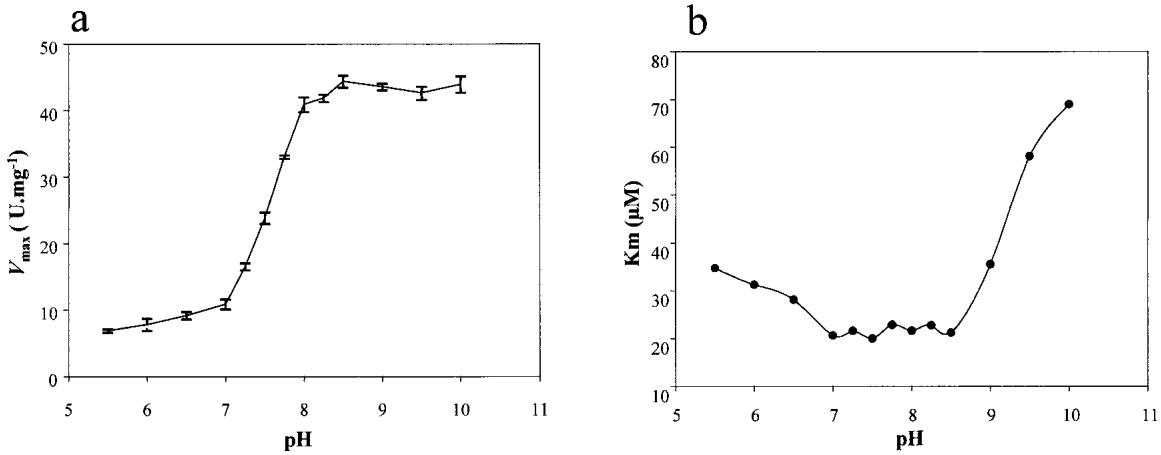

Fig. 4. Effect of pH on K_m and V_{max}. Data were obtained using different pH indicator/buffer pairs at 50/2500 μM at 25 °C. (*a*) Effect of pH on V_{max}. (*b*) Effect of pH on K_{mapp}. Three pK_a values (7.1, 8.8, and 10) were obtained by fitting the K_m data to the equation: $K_{mapp} = K_m([H^+]/K_1 + 1 + K_2/[H^+]\{ 1+ K_3/[H^+]\})$, using a K_m value of 2.35 μM. The concentration of $MgCl_2$ was 25 mM.

tivity was exponential. Linear fitting of data to the Arrhenius equation (Cornish-Bowden et al. 1995) gave a value for the activation energy of 12,500 cal mol^{-1}.

Metal ion requirement

In the absence of $MgCl_2$, *L. major* dUTPase was totally inactive. The V_{max} value increased with increasing concentrations of Mg^{2+} from 14 units mg^{-1} at 0.5 mM to 40 units mg^{-1} at 25 mM. This latter value remained constant at higher Mg^{2+} concentrations. No change in the apparent K_m was observed when the Mg^{2+} concentration was raised from 0.5 to 250 mM.

No enzymatic activity was observed when Mg^{2+} was replaced by Ca^{2+} or Cu^{2+}. However, the addition of Mn^{2+} or Co^{2+} instead of Mg^{2+} to the reaction mixture produces an increase in K_m between 2- and 4-fold the value obtained with Mg^{2+}, whereas V_{max} remained unaltered.

The pH variation during the reaction was different depending on the metal ion used because the increase in the absorbance measured was different. This observation suggests that the nature of the ion used in the reaction has an important role in the number of protons released during the hydrolysis of dUTP.

Hydrolysis of other nucleotides

The hydrolysis of different nucleotides by *L. major* dUTPase was studied under the same conditions as used in the measurements of dUTP hydrolysis. The enzyme assay was carried out using as substrates dTTP, dCTP, dATP, and dGTP at a final concentration of 500 μM, and 0.3 μM of dUTPase. All nucleotides tested were inefficiently hydrolyzed compared to the substrates dUTP and dUDP. Table 1 shows the K_{mapp} and k_{cat} values obtained with the different nucleotides. The rate of hydrolysis of UTP was extremely low; the K_m value was higher than 2500 μM, and the specificity constant lower than 2×10^3 M^{-1}s^{-1}.

Inhibition

We have characterized in detail strong competitive product inhibition of dUTPase by dUMP. Additionally, two substrate analogs have been tested as possible inhibitors of *Leishmania* dUTPase. First DMT-dU, an efficient inhibitor of *E. coli* dUTPase, was analyzed at concentrations from 10 to 1000 μM. Different K_{mapp} values were obtained, whereas the V_{max} values were constant, indicating competitive inhibition with a K_i value higher than 1000 μM. Studies were also performed with α-β-imido-dUTP, using concentrations from 0.3–6 μM, which proved to be an effective inhibitor of the enzyme with a K_i value of 0.89 μM. (Fig. 5). Both K_i values were obtained taking into account product inhibition. No hydrolysis of these inhibitors could be detected after overnight incubations with 0.3 μM dUTPase.

Table 1. *Catalytic parameters* K_m *(μM),* k_{cat} *(s^{-1}) and specificity constant ($M^{-1}s^{-1}$) of* Leishmania major *dUTPase for different nucleoside triphosphates and UTP*

Substrate	K_m	k_{cat}	k_{cat}/K_m
dUTP	2.11	49	2.3×10^7
dATP	1034	5.4	5.1×10^3
dTTP	1514	8.6	5.5×10^3
dGTP	> 2000	6.4	6.4×10^3
dCTP	> 2500	13	5.0×10^3
UTP	> 2500	<0.5	$< 2 \times 10^3$

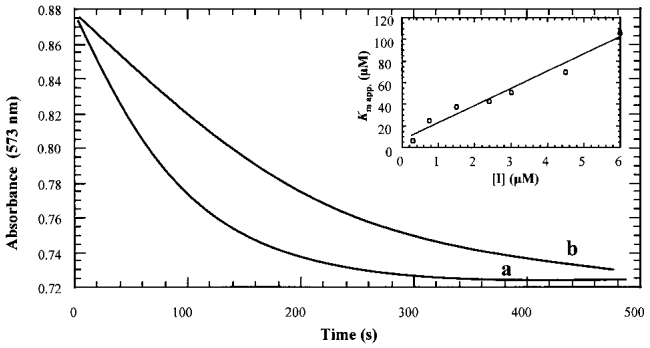

Fig. 5. Inhibition of *Leishmania major* dUTPase by α-β-imido-dUTP. Hydrolysis was performed using 30 nM enzyme at pH 8 and 25°C with (*a*) 25 μM dUTP and no inhibitor, and (*b*) 25 μM dUTP and 10 μM α-β-imido-dUTP. Inset linear dependence of apparent K_m on inhibitor concentration.

Discussion

Protozoan dUTPases differ profoundly from general eukaryotic dUTPases in amino acid sequence and presumably structural features. Previous studies have been carried out with bacterial, viral, and human dUTPases (Bjornberg and Nyman 1996; Larsson et al. 1996a,b; Nord et al. 1997). These enzymes are homotrimers or monomers and are highly specific for dUTP. In the present study we perform an in-depth kinetic characterization with a dimeric trypanosomatid dUTPase and compare results with the corresponding data available at the moment for homotrimeric and monomeric dUTPases.

The kinetic constants for the hydrolysis of dUTP are similar to other dUTPases, with a low K_m for dUTP, and a high specificity constant. Conversely, the estimated k_{cat} for dUTP hydrolysis, obtained from single-turnover experiments, was higher than other dUTPases described so far. This k_{cat} was two-fold higher than the k_{cat} obtained using V_{max}, which might be attributable to a "burst effect" caused by the high product (dUMP) inhibition, which slows down the reaction in multiple turnover experiments.

One of the main peculiarities of *L. major* dUTPase is the capacity of hydrolysis of dUDP. The K_m was 30-fold higher, suggesting lower binding to the active site than in the case of the nucleoside triphosphate although the specificity constant was similar to the value obtained for dUTP. This characteristic is common only to the T2-T4 phage dCTPase-dUTPase, whereas dUDP is a strong competitive inhibitor for the rest of dUTPases with K_i values between 3 and 17 μM. In fact the *E. coli*, EIAV, and human dUTPases have been crystallized in complex with this nucleoside diphosphate (Larsson et al. 1996a,b; Prasad et al. 2000), which it is not hydrolyzed. The biological role of dUDPase activity may be to assure production of dUMP, the substrate of thymidylate synthase. It is also possible that intracellular levels of dUDP are high in these organisms and that this nucleotide is readily converted to dUTP, which is toxic to cells. Hydrolysis of dUDP would further ensure low dUTP levels and reduced incorporation of uracil into DNA.

Regarding the products of the reaction, the pyrophosphate activity measurements confirm that in the hydrolysis of dUTP, the products are dUMP and PP_i. However, although dUMP acts as a strong competitive inhibitor, PP_i does not inhibit the reaction as in other organisms (Larsson et al. 1996a,b), and the K_{ip} value for dUMP is ~10-fold lower in the case of the *Leishmania* enzyme. This observation might have interesting biological implications and further studies would be of interest to determine the possible influence of the dUMP pool and its utilization by thymidylate synthase on dUTPase activity.

The analysis of the influence of pH on V_{max} suggests that the enzyme might have a residue important for activity with a pK_a of ~7–8. Conversely, the pH dependence of K_m shows that formation of the enzyme-substrate complex requires deprotonation of a group exhibiting a pK_a of about 7.1. Taking into account these observations, a general base catalysis mechanism for the hydrolysis of the substrate by dUTPase mediated by a histidine residue could be postulated. The dependence of K_m also gives a pK_a value of 10 suggesting that a tyrosine residue could be involved in substrate binding. There are several candidates that might correspond to the mentioned residues such as His-82 or Tyr-191 (Camacho et al. 1997). Thus, for instance Tyr-191 is present in a consensus sequence that appears in the uridine-binding site of enzymes such as pseudouridin synthetase (Kooning 1996). Site-directed mutagenesis is underway to confirm these observations.

The importance of the metal ion in the hydrolysis of dUTP has been demonstrated by the finding that metal-free dUTP is not hydrolyzed by *E. coli* dUTPase and that Mg^{2+} enhances the binding of dUTP by a factor of 100 (Larsson et al. 1996a,b). For instance, optimal activity values are reached at 50 mM of $MgCl_2$ in the case of the enzymes of *Bacillus subtilis* and HSV (Price and Frato 1975). *L. major* dUTPase presents a maximum of activity at 25 mM of Mg^{2+} and almost the same V_{max} values were obtained with $Mn^{2+}$and Co^{2+} at the same concentration. With the latter ions, the K_m values were 2- and 3-fold higher, respectively, suggesting that the metal ion might play an important role in the binding of dUTP to the active site, although as indicated for the human enzyme, this requirement could be purely structural (Mol et al. 1996). Accordingly, Ca^{2+} could not substitute Mg^{2+} in catalysis, probably attributable to the greater bulk of this ion, which can disturb the binding of the substrate to the active site, avoiding the hydrolysis. *L. major* dUTPase can discriminate easily between dUTP and the other common dNTPs, thus it is almost exclusively specific for dUTP, showing a specificity constant three orders of magnitude higher for this nucleotide than for the rest of substrates tested. The next best substrates were dATP and

dTTP. Assuming that the association rate constant of enzyme and substrate is equal in bacterial, human, and viral dUTPases, and using the K_m values for dUTP hydrolysis to normalize inhibitor binding by the different enzymes, the binding discrimination between dUTP and other dNTPs would be over 30 times greater in these dUTPases than in the case of the *L. major* dUTPase. The fact that this enzyme can discriminate not only the uracil moiety from other closely related bases, but also the deoxyribose moiety from ribose is essential to prevent a wasteful and possibly fatal hydrolysis of nucleotides needed for synthesis of DNA and RNA.

Our results show that *Leishmania* dUTPase has a slow turnover and an almost optimized specificity constant, characteristics which are common to all the dUTPases described to date. The similar kinetic properties suggest that the enzyme is equivalent in function and would refute the hypothesis that a slow turnover and good specificity constant are essential for an almost exclusive transfer of dUMP from dUTP.

The strong competitive inhibition properties of the triphosphate analog α-β imido-dUTP have been analyzed in different dUTPases like *E. coli* (Larsson et al. 1996b), HSV-1 (Nord et al. 1997), and EIAV (Nord 2000). As shown in Table 2, in the case of *L. major* dUTPase, the K_i value was considerably low, and in consequence the selectivity indexes relative to the rest of the eukaryotic enzymes were >10. These data suggest that a pronounced requirement for a triphosphate moiety to form a catalytic complex is necessary and provides support for the differences in the architecture of the active sites between dUTPases of trypanosomatids and other organisms. In addition, the lack of inhibition in the case of the *Leishmania* enzyme by the DMT-dU compared to the low K_i described for the *E. coli* enzyme (Persson et al. 1996), denotes possible structural differences between both dUTPases and the possibility of a specific inhibition of the parasitic enzyme.

In summary, dimeric *L. major* dUTPase does not exhibit any of the five consensus motifs present in the bacterial, viral, and human enzymes (Camacho et al. 2000) but displays comparable kinetic parameters for dUTP hydrolysis with marked dissimilarities in the binding of dUDP and dUMP, suggesting differences in the structure of the active sites. Diffractable material has been obtained in crystallization trials of *T. cruzi* dUTPase, another parasitic enzyme that displays a 55% identity with *L. major* (Bernier-Villamor et al. 1999). If structural information is made available, it may be exploited in the design of specific nucleotide analogs potentially useful for the medical treatment of leishmaniasis.

Materials and methods

Chemicals

2′-dUTP, 2′-dTTP, 2′-dCTP, and UTP were purchased from Pharmacia. Sodium salts of 2′-dCDP, 2′-dUDP, 2′-dUMP, 2′-dATP, and 2′-dGTP, (DMT-dU), the "pyrophosphate reagent," bovine albumin (BSA), $CaCl_2$, $MnCl_2$, $MgCl_2$, $CuCl_2$, $CoCl_2$, and the pH indicators cresol red, alyzarin yellow, bromocresol purple, and bromothymol blue were from Sigma. The buffer N, N-bis (2-hydroxyethyl) glycine (BICINE) was obtained from USB. Recombinant enzyme was overexpressed and purified to homogeneity following the protocol described by Camacho et al. (2000) and stored in 50 mM BICINE at pH 8.0 and 25 mM $MgCl_2$ at −80°C. The monodisperse and nonionic detergent octaethylene glycol mono-n-dodecylether ($C_{12}E_8$) was obtained from Nikko Chemicals. All the concentrations of the nucleotides and the enzyme were calculated spectrophotometrically (HP-8453, Hewlett Packard) at 280 nm, using the extinction coefficient ($\varepsilon_{280\ nm} = 1.75$ ml $mg^{-1}cm^{-1}$). The purity of the nucleotides was assessed by a MonoQ column attached to an FPLC (Pharmacia LKB), equilibrated with 0.01 M HCl in 0.01 M KCl and elution performed with a 20 ml gradient of 0.01 M to 0.2 M KCl in 0.01 HCl. Other chemicals used in these experiments were of the highest quality available.

Table 2. *Michaelis-Menten, specificity and inhibition constants of viral (EIAV, HSV-1 and MMTW), bacterial (*E. coli*) and* Leishmania major *dUTPases towards different nucleoside triphosphates and uracil triphosphate analogues*

Substrate/inhibitor	*L. major*	*E. coli*[a]	EIAV[b]	HSV-*l*[c]	MMTV[d]
dUTP: $K_m(k_{cat}/K_m)$	2.11 (2.3×10^7)	0.2 (4×10^7)	1.1 (2×10^7)	0.3 (2×10^7)	0.8 (2×10^6)
dTTP: $K_m(k_{cat}/K_m)$	1514 (5.5×10^3)	> 20000 (34)	260 (< 2000)	400 (1000)	N.D. (2000)
dCTP: $K_m(k_{cat}/K_m)$	> 2500 (5.0×10^3)	4000 (< 100)	3000 (1000)	1000 (2000)	N.D.
UTP: $K_m(k_{cat}/K_m)$	> 2500 (< 2×10^3)	2500 (< 100)	N.D.	1000 (200)	N.D.
dUDP: $K_m(k_{cat}/K_m)$	62.70 (1×10^6)	$K_i = 15$	$K_i = 3.6$	$K_i = 17$	N.D.
dUMP: K_{ip}	13.05	1500	130	170	N.D.
α-β-imido-dUTP: K_i	0.89	5	0.6	0.9	N.D.

The units for K_m and K_i *are μM and for* k_{cat}/K_m $M^{-1}s^{-1}$. All values were obtained at pH 8.
[a] Larsson et al. 1996b.
[b] Nord et al. 1997.
[c] Bergman et al. 1995.
[d] Bjornberg and Nyman 1996.

Kinetic measurements

Nucleotide hydrolysis was monitored by mixing enzyme and substrate with a rapid kinetic accessory (Pharmacia) attached to a spectrophotometer (HP-8453, Hewlett Packard) and connected to a computer for data acquisition and storage. The measurements were performed at 25°C, and the solutions containing 25 mM $MgCl_2$ and 1 mg ml^{-1} BSA or 0.01% $C_{12}E_8$ (w/v) were degassed previously. Protons, released through the hydrolysis of nucleotides, were neutralized by a pH indicator in a weak buffered medium with similar pK_a and monitored spectrophotometrically at the absorbance peak of the basic form of the indicator. The ratio between the indicator and the buffer concentration was 50:2500 (μM), but it depended on the number of protons expected to be released in the reaction, the buffer capacity of the solution, and the extinction coefficient of the pH indicator. Absorbance changes were kept within 0.1 units. The indicator/buffer pairs were bromocresol purple/MES (pH 5.7–6.2, 605 nm), bromothymol blue/MOPS (pH 6.5–7.3, 622 nm), cresol red/BICINE (pH 7.5–8.5, 573 nm), and alyzarin yellow/CHES (pH 9.0–10.0, 445nm). Prior to each experiment, the apparent pK_a values of each indicator/buffer pair were determined by titration, in the stopped-flow spectrophotometer, of solutions identical to those used in the kinetic experiments but without enzyme and substrate. The final enzyme concentration in multiple-turnover assays was 0.03 μM, 10–300 μM dUTP, 25 mM $MgCl_2$ and the ionic strength 0.1 M, at pH 8. The majority of experiments were performed at 25°C. Indicator absorbance changes corresponding to complete hydrolysis of nucleotides were recorded in the computer, and the kinetic parameters V_{max} and K_{mapp} (or slope) were calculated by fitting the data to the integrated Michaelis–Menten equation, adapted to enzymes which have an appreciable affinity for the product. The decrease in velocity with time is due to the decrease of saturation of the enzyme with substrate and the increase of the product inhibition: $[P]/t = -K_m\{(K_{ip} + [S]_0)/(K_{ip} - K_m)\}(2.3/t \log[S]_0/[S]) + V_{max}/(1 - K_m/K_{ip})$, where $K_{mapp} = -K_m\{(K_{ip} + [S]_0)/(K_{ip} - K_m)\}$ (Segel 1975). 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_{ip} for product inhibition, the obtained slopes from the plots of the integrated velocity equation (or K_{mapp}s obtained at different substrate concentrations) were replotted versus $[S_0]$. Units for V_{max} were μmol min^{-1}.

Single-turnover measurements were performed by reacting fourfold excess dUTPase (2–32 μM) with dUTP (0.5–8 μM), in the presence of cresol red (0.5 mM) and BICINE (25 mM), using the stopped-flow spectrophotometer at 25°C and a fixed concentration of 25 mM $MgCl_2$. Instead of BSA we used the detergent $C_{12}E_8$, because of the interference in the absorbance at short times of BSA. The rate constant observed for the exponential absorbance traces are given by the equation $k_{obs} \approx k_{cat}/(1 + K_m/C_e)$, as described by Larsson et al. (1996a,b). When the enzyme concentration (Ce) is at least four times the substrate concentration and $C_e >> K_m$ it is assumed that $k_{obs} \cong k_{cat}$.

PP_i release

The "pyrophosphate reagent" (Sigma, ref. P7275) for enzymatic determination of pyrophosphate, was utilized to quantify spectrophotometrically the amount of PP_i produced in the hydrolysis of dUTP by dUTPase, as described by the manufacturer. The amount of pyrophosphate released was determined using a coupled enzyme system containing fructose-6-phosphatase kinase (pyrophosphate-dependent), aldolase, triosephosphatase isomerase and α-glycerophosphate dehydrogenase. Two moles of NADH are oxidized to NAD^+ per mole of pyrophosphate consumed. The reaction is monitored spectrophotometrically at 340 nm. Measuring the increase of the absorbance of a blank containing only water, and the increase due to the PP_i released after the incubation of 2 μM dUTPase with 25 μM dUTP at 30°C during 5 min, the amount of PP_i in the sample can be calculated with the equation: PP_i (μmol ml^{-1}) = $(\Delta A_{TEST} - \Delta A_{BLANK})$ 4.82 (O'Brien 1976).

Metal ion requirements

To study the metal ion requirements of *L. major* dUTPase, different amounts of $MgCl_2$, $MnCl_2$, $CaCl_2$, $CuCl_2$, and $CoCl_2$ were added to reaction mixtures containing 2500 μM BICINE, 50 μM cresol red, and 1 mg ml^{-1} of BSA. The reaction was measured at 25°C and pH 8, using the same stopped-flow spectrophotometer.

Inhibition

The inhibition of *L. major* dUTPase by DMT-dU and α-β-imido-dUTP was determined by including the inhibitor at various concentrations in the assay mixture (50μM cresol red, 2500 μM BICINE, 0.03 μM dUTPase, and 50 μM dUTP). All the inhibition reactions were performed at 25 °C and pH 8. For each inhibitor concentration, the apparent K_{mapp} value was obtained using the integrated Michaelis–Menten equation as described for dUTP. K_m values are a linear function of the inhibitor concentration in competitive systems with product inhibition: $K_{mapp} = K_m/K_i/(1 - K_m/K_{iproduct})[I] + K_m*(1 + [S_0]/K_{iproduct})/(1 - K_m/K_{iproduct})$. 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. This way the K_i value for the inhibitor can be obtained from the slope value of the linear plot of K_{mapp} versus [I] and takes into account product inhibition (Segel 1975).

The purity of the inhibitor was evaluated on a Mono Q column, using as starting buffer 5 mM PO_4H_2Na at pH 7 with 50 mM NaCl and 5 mM PO_4H_2Na with 350 mM NaCl as elution buffer. The flow applied was 1 ml min^{-1}. The hydrolysis of the inhibitor was monitored in the same way but analyzing a mixture of dUTPase and inhibitor after an overnight incubation.

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