Properties of *Leishmania major* dUTP nucleotidohydrolase, a distinct nucleotide-hydrolysing enzyme in kinetoplastids

Ana CAMACHO, Fernando HIDALGO-ZARCO, Victor BERNIER-VILLAMOR, Luis M. RUIZ-PÉREZ and Dolores GONZÁLEZ-PACANOWSKA[1]

Instituto de Parasitología y Biomedicina 'López-Neyra', Consejo Superior de Investigaciones Científicas, Calle Ventanilla 11, 18001-Granada, Spain

We have previously reported the presence, in the parasitic protozoan *Leishmania major*, of an enzyme involved in controlling intracellular dUTP levels. The gene encoding this enzyme has now been overexpressed in *Escherichia coli*, and the recombinant enzyme was purified to homogeneity. Biochemical and enzymic analyses of the *Leishmania* enzyme show that it is a novel nucleotidohydrolase highly specific for deoxyuridine 5′-triphosphate. The enzyme has proved to be a dimer by gel filtration and is able to hydrolyse both dUTP and dUDP quite efficiently, acting as a dUTP nucleotidohydrolase (dUTPase)-dUDP nucleotidohydrolase but has a limited capacity to act upon other nucleoside di- or triphosphates. The reaction products are dUMP and PP_i when dUTP is the substrate and dUMP and P_i in the case of dUDP. The enzyme is sensitive to inhibition by the reaction product dUMP but not by PP_i. dUTPase activity is highly dependent on Mg^{2+} concentrations and markedly sensitive to the phosphatase inhibitor, NaF. In summary, *Leishmania* dUTPase appears to be markedly different to other proteins characterized previously that accomplish the same function.

Key words: dUTPase, uracil.

INTRODUCTION

The enzyme dUTP nucleotidohydrolase (dUTPase, EC 3.6.1.23) catalyses the hydrolysis of dUTP to dUMP and PP_i. It is involved in the removal of dUTP from the dNTP pool, thus preventing this nucleotide from being available for DNA polymerase and therefore all the consequences resulting directly from the incorporation of uracil in DNA [1]. Futhermore, it plays an essential role in *de novo* biosynthesis of dTTP by furnishing the substrate for thymidylate synthase. 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. The inability to isolate viable dUTPase-null mutants in both *Escherichia coli* [2] and *Saccharomyces cerevisiae* [3] infers the essential role of this enzyme for the viability of these organisms.

Kinetic properties of dUTPase from *E. coli* have been characterized recently using a spectrophotometric method for the continuous measurement of the reaction [4]. This enzyme is highly specific for dUTP, with a specificity constant 10^5 times higher than for any of the other common nucleotides. The Mg^{2+} ion has been reported to enhance the binding of dUTP to dUTPase by a factor of 100.

The three-dimensional structures of dUTPases from *E. coli* [5,6], human [7], feline immunodeficiency virus (FIV) [8] and equine infectious anaemia virus (EIAV) [9] have been determined and show that the enzyme is a trimer of identical subunits, with three active sites, each of which is formed by the contribution of residues from each subunit [6,7].

The parasitic protozoan *Leishmania major* has been shown to encode a protein with dUTPase activity that can substitute efficiently the *E. coli* enzyme [10]. The amino acid sequence is markedly different from all eukaryotic dUTPases characterized so far, although there is a certain sequence homology with dCTPase of the bacteriophage T4 [11]. This paper reports the purification and characterization of *L. major* dUTPase from *E. coli* BL21(DE3) cells in which the gene has been overexpressed. Substrate specificity, cation requirement, molecular mass and kinetic behaviour have been established. Furthermore, the present data show that the *Leishmania* enzyme not only has certain structural and molecular features that are unique to these organisms but also differs in its hydrolytic capacity from all the enzymes so-far characterized accomplishing the same function.

EXPERIMENTAL

Materials

Deoxy[5-^{3}H]UTP (15 μCi/nmol) ammonium salt was purchased from Amersham. Poly(ethyleneimine) plates for TLC with a fluorescent marker, sodium salts of 2′-dCDP, 2′-dUDP, 2′-dUMP, 2′-dATP and 2′-dGTP, NaF, the phosphorus inorganic quantitative colorimetric determination system, NaCl, $CaCl_2$, $MnCl_2$, $MgCl_2$ and dithiothreitol (DTT) were from Sigma. 2′-dUTP, 2′-dTTP, 2′-dCTP and UTP were purchased from Pharmacia. PMSF, trizma base and leupeptin were purchased from Boehringer Mannheim. Molecular-mass markers for SDS/PAGE, acrylamide, bisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylenediamine and ammonium persulphate were purchased from Bio-Rad.

Expression system

The expression-system construction was reported previously [10]. The expression plasmid, named pETLMDUT, was used to transform the bacterial strain BL21(DE3), carrying the gene for T7 RNA polymerase under the control of the inducible *lacUV5* promoter [12]. Bacterial clones transformed with pETLMDUT were grown in Luria–Bertani medium containing 100 $\mu g \cdot ml^{-1}$ ampicillin at 37 °C. Upon reaching an A_{600} of 0.6, 1 mM isopropyl

Abbreviations used: dUTPase, dUTP nucleotidohydrolase; dUDPase, dUDP nucleotidohydrolase; DTT, dithiothreitol; EIAV, equine infectious anaemia virus; HSV, herpes simplex virus.

[1]To whom correspondence should be addressed (e-mail dgonzalez@ipb.csic.es).

β-D-thiogalactoside was added, and cultures were grown for an additional 3.5 h. Cells were collected by centrifugation and stored at −80 °C until use.

Protein purification

A frozen pellet from 2 litres of *E. coli* BL21(DE3)/pETLMDUT cells overproducing *L. major* dUTPase was thawed and resuspended in 20 ml of a buffer containing 25 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM DTT, 0.2 mM PMSF and 20 $\mu g \cdot ml^{-1}$ leupeptin. Cells were sonicated and centrifuged at 12000 ***g*** for 30 min. The supernatant was used in the purification procedure. DEAE–cellulose DE52 (Whatman) was pretreated according to the manufacturer. Hydroxyapatite Bio-Gel™ HTP gel was purchased from Bio-Rad. Protein elution was monitored using the Bradford protein assay [13]. In gel-filtration experiments, a Superdex 75 HR 10/30 column was attached to an ÄKTA purifier 10 system (Pharmacia Biotech).

Active fractions were analysed for dUTPase activity by measuring the production of [^{3}H]dUMP from [^{3}H]dUTP [14]. Development of the reaction mixtures in TLC plates was performed as described by Beardsley and Abelson [15]. Reactions containing 150 mM Tris/HCl, pH 7.5, 6 mM β-mercaptoethanol, 3 % (w/v) BSA, 25 mM $MgCl_2$ and 10 nM purified enzyme in a final volume of 20 μl were started by the addition of 0.1 mM dUTP and carried out at 37 °C for 10 min after 5 min of preincubation at the same temperature. The formation of dUMP was stopped by addition of EDTA to a final concentration of 0.3 M. A unit of dUTPase activity was defined as the amount of enzyme required to hydrolyse 1 μmol of dUTP per min at 37 °C.

When studying the hydrolysis of unlabelled nucleotides, the reaction mixture contained 150 mM Tris/HCl, pH 7.5, 6 mM β-mercaptoethanol, 5 mM $MgCl_2$, 0.1 M dNTP and 50 nM purified protein in a final volume of 200 μl. Incubations were carried out at 37 °C for 30 min and were stopped by boiling for 3 min followed by centrifugation (13000 ***g*** for 5 min) to remove insoluble material. Samples were applied to a MonoQ™ 5 HR 10/30 column and analysed at A_{254}. The column was pre-equilibrated with a 5 mM sodium phosphate buffer, pH 7.5. After washing the column with three bed volumes of equilibrating buffer, elution was performed using a 12-ml linear NaCl gradient between 0.05 and 0.35 M in the same buffer, at a flow rate of 0.3 $ml \cdot min^{-1}$. Nucleotides in the assay were identified by comparison of retention times with those of individual nucleotide standards.

In order to analyse dUDP hydrolysis, unlabelled dUDP at a final concentration of 100 μM was used as substrate for the dUTPase assay. The reaction was carried out at 37 °C for 30 min in a final volume of 1 ml containing 150 mM Tris/HCl, pH 7.5, 6 mM β-mercaptoethanol, 5 mM $MgCl_2$ and 10 nM purified enzyme. Samples (200 μl) were taken from the total mixture at fixed time intervals of 5 min, and the reactions were terminated by boiling, followed by centrifugation. The reaction products were analysed using the same conditions as indicated above for unlabelled nucleotides. dUDP nucleotidohydrolase (dUDPase) activity was calculated by monitoring the rate of decrease in the area of the dUDP peak.

A standard colorimetric assay was performed to detect P_i during the course of the reaction. PP_i was converted to P_i by incubation with inorganic pyrophosphatase. The amount of PP_i was calculated as one-half of the P_i measured in the Fiske–Subbarow [15a] assay.

Cross-linking assay

Purified *Leishmania* dUTPase (0.1 $mg \cdot ml^{-1}$) was incubated at 37 °C in 50 mM Hepes, pH 8.0, 0.1 mM EDTA, 10 mM $MgCl_2$ and 0.5 mM DTT in the presence or absence of 100 μM disuccinimidyl suberate. Aliquots of 50 μl were collected at different time intervals and the reactions were stopped by the addition of an equal volume of 50 % trichloroacetic acid followed by two washing steps with acetone. The resulting pellets were finally dissolved in sample buffer and analysed in 12 % polyacrylamide gels by SDS/PAGE.

RESULTS

Purification of *L. major* dUTPase

Recombinant dUTPase was purified using a combination of adsorption and anion-exchange chromatography. Briefly, the soluble extract was applied to a 30-ml hydroxyapatite column (1.5 × 30 cm) equilibrated with buffer A (20 mM K_2HPO_4, pH 6.5, 5 mM $MgCl_2$ and 2 mM DTT). Enzyme was eluted with a 300-ml linear gradient of 20–200 mM K_2HPO_4. *L. major* dUTPase eluted from the hydroxyapatite column between 50 and 100 mM K_2HPO_4. The fractions containing the enzyme were diluted in buffer B (20 mM K_2HPO_4, pH 7.5, 5 mM $MgCl_2$ and 2 mM DTT), concentrated and applied further to a 30-ml DEAE–cellulose column (1.5 × 30 cm) equilibrated with buffer B. The protein was eluted with a 300-ml linear gradient of NaCl (0–200 mM) in the same buffer, at a flow rate of 1 $ml \cdot min^{-1}$. dUTPase eluted at 40 mM NaCl. Fractions with enzyme activity were pooled, concentrated and frozen at −80 °C with 100 mM $MgCl_2$. The dUTPase preparation finally obtained had a specific activity of 44 ± 4.45 units $\cdot mg^{-1}$, and was close to homogeneity. Using this procedure, the enzyme was purified approx. 3.7-fold with an average yield of 44 %. The purification scheme is summarized in Table 1. Enzyme purity was assessed by SDS/PAGE and found to be greater than 99 % pure by scanning densitometry (Figure 1).

Molecular properties

The molecular mass of *Leishmania* dUTPase deduced from the amino acid sequence was reported previously to be 31.42 kDa [10]. In order to establish the subunit organization of the native enzyme, a mixture containing reference proteins and purified dUTPase at a final concentration of 1 $mg \cdot ml^{-1}$ was diluted in buffer C (50 mM K_2HPO_4, pH 7.0, and 150 mM NaCl) and loaded on to a Superdex 75 HR 10/30 gel-filtration column using a flow rate of 0.5 $ml \cdot min^{-1}$. The enzyme eluted at 18.70 min, between the peaks of BSA and ovalbumin, which corresponds to an apparent molecular mass of 62848.16 Da, thus indicating that *Leishmania* dUTPase behaves as a dimer in gel filtration. Fractions corresponding to this single protein peak in the chromatogram were the only ones with dUTPase activity. Certain enzymes such as EIAV dUTPase [16] have been analysed by gel filtration in the presence or absence of Mg^{2+} in the elution buffer and the same elution profile, corresponding to a trimeric organization, is always obtained. These observations suggested that divalent cations are not required for subunit association. Gel filtration of *Leishmania* dUTPase was also carried out with prior incubation of the enzyme with 5 mM dUTP and using elution buffer D containing Mg^{2+} (0.05 M Tris/HCl, pH 8.5, and 100 mM $MgCl_2$). Again only a peak of activity corresponding to a dimeric form of the protein was obtained. Cross-linking and SDS/PAGE analysis (Figure 2) also confirmed the subunit arrangement of the enzyme. Hence, unlike other eukaryotic, prokaryotic and phage dUTPases, the active form of the *L. major* enzyme appears to be a dimer.

Table 1 Purification of *L. major* dUTPase

Fraction	Total protein (mg)	Total units	Specific activity* (units · mg^{-1})	Yield (%)	Purification (-fold)
Crude extract	126.4	1491.52	11.88 ± 1.5	100	1
Hydroxyapatite	25.55	1120.11	43.84 ± 2.25	75.1	3.69
DEAE–cellulose	14.80	652.09	44.06 ± 4.45	43.71	3.7

* Results are means ± S.E.M. for dUTPase activities determined in three independent incubations.

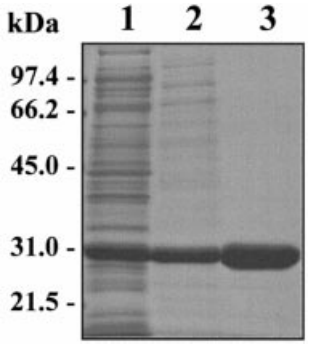

Figure 1 SDS/PAGE of *Leishmania* dUTPase

Lane 1 corresponds to the isopropyl β-D-thiogalactoside-induced crude extract from BL21(DE3)/pETLMDUT cells, lane 2 shows the protein eluted from the hydroxyapatite column and lane 3 corresponds to the protein from the pooled peak fractions obtained after DEAE–cellulose chromatography.

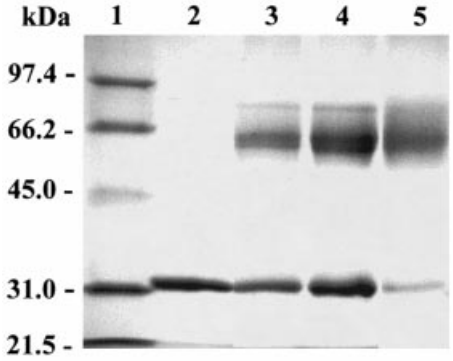

Figure 2 SDS/PAGE analysis of cross-linked *Leishmania* dUTPase

Lane 1 corresponds to molecular-mass standards. Lane 2 corresponds to enzyme incubated in reaction buffer for 120 min in the absence of cross-linking agent. Lanes 3–5 show the reaction products generated after incubation with disuccinimidyl suberate for 30, 60 and 120 min respectively.

Effect of cations and NaF

A common characteristic shared by dUTPases found in different organisms is their dependence on Mg^{2+} or Mn^{2+} for optimal activity. The radioactive assay was performed in the presence of different concentrations of metal ions to determine their effect on *Leishmania* dUTPase. Ca^{2+}, Mn^{2+}, Mg^{2+} and Na^{+} were added to the reaction mixture at 25 and 100 mM. Optimal levels of activity were only obtained when the added divalent cations were Mg^{2+} or Mn^{2+}. Neither Ca^{2+} nor Na^{+} supported measurable activity. The effect of different $MgCl_2$ concentrations was studied in detail. Added Mg^{2+} stimulated enzyme activity, reaching maximum values at 25 mM.

Table 2 Inhibition of dUTPase by NaF

NaF (mM)	Specific activity (units · mg^{-1})*	Inhibition (%)
–	41.31 ± 1.78	–
0.005	31.77 ± 1.85	23.09
0.02	25.60 ± 0.14	38.02
0.2	11.29 ± 0.35	72.67
2	3.83 ± 0.99	90.72
50	2.41 ± 0.01	94.16
200	0.48 ± 0.15	98.83

* Results are means ± S.E.M. for dUTPase activities determined in three independent incubations.

Most dUTPases described have been reported to be active in the presence of NaF, a potent non-specific phosphatase inhibitor [17–19]. In the case of the *Leishmania* enzyme, activity was markedly sensitive to fluoride ions, and was inhibited by 73% by the addition of 0.2 mM NaF under standard conditions, as shown in Table 2. A similar sensitivity has been reported previously for the phage-induced dCTPase-dUTPase [20].

Hydrolysis of nucleotides

The hydrolysis of different nucleotides by *Leishmania* dUTPase was studied. The enzyme assay was carried out using as substrates the unlabelled nucleotides: dUTP, dUDP, dATP, dGTP, dTTP, dCTP and dCDP. Table 3 shows the hydrolysis capacity under conditions in which conversion of dUTP to dUMP continues until completion. Of all the nucleotides tested, only dUTP and dUDP were hydrolysed efficiently. Whereas dUDP is clearly hydrolysed by the protozoan enzyme, this NDP has been reported to be an inhibitor for *E. coli* dUTPase, with a K_i of 15 μM, but not a substrate. It will bind to the enzyme without being hydrolysed, preventing the binding of the real substrate, dUTP [4]. NDP hydrolase activities have been reported previously for a dUTPase described in Yoshida sarcoma cells that hydrolyses both dUTP and dUDP [19] and for the dCTPase-dUTPase induced in *E. coli* cells by the T4 phage, which is active with both dUDP and dCDP [20].

PP_i release

The nature of the products formed during the enzymic hydrolysis of dUTP and dUDP was determined in standard colorimetric assay mixtures where the substrate concentration was increased 1000-fold. Two reactions were run in parallel for each substrate except for the omission of inorganic pyrophosphatase in one of them. HPLC analysis of the reaction products shows that disappearance of both dUTP and dUDP is accompanied by the appearance of dUMP (Figure 3). Data in Table 4 show that no P_i was detected in the reaction mixture after incubation with dUTP (1.5 mM), while in reactions with inorganic pyrophosphatase proportional amounts of P_i were measured (with 100% hydrolysis of dUTP to dUMP and PP_i). However, in the case of incubations with dUDP as a substrate of *Leishmania* dUTPase, the products of hydrolysis were P_i and dUMP. Hence, the enzyme acts upon the α–β bond of nucleoside di- and

Table 3 Hydrolysis of nucleotides by recombinant *L. major* dUTPase

	dUTP	dTTP	dCTP	dATP	dGTP	dUDP	dCDP
Hydrolysis (%)	96.29	6.8	4.91	30.14	6.13	100	16.51

triphosphates releasing respectively P_i and PP_i as products of the reaction. Accordingly, the *Leishmania* enzyme should be considered as a dUTPase-dUDPase.

Kinetics

Standard procedures were used to determine kinetic parameters. Initial velocity studies regarding dUTP hydrolysis were determined at 37 °C, keeping Mg^{2+} at a fixed concentration (25 mM) and varying the dUTP concentration between 0.5 and 500 μM with purified enzyme present at a final concentration of 1 nM. K_m and V_{max} values were obtained by a non-linear least-squares fit of the data to the Michaelis–Menten equation. The K_m value obtained this way for dUTP was 4.5 ± 0.5 μM and the V_{max} was 41.0 ± 1.0 units·mg^{-1}. For determining the rate for dUDP hydrolysis, the enzyme was incubated in the presence of different concentrations of unlabelled dUDP and samples from the total reaction mixture were analysed at different intervals on a MonoQ column as described in the Experimental section. Integration of the peaks corresponding to the products obtained after incubation at different substrate concentrations allowed us to determine the approximate kinetic constants for the hydrolysis of dUDP. Thus the K_m value obtained this way was estimated to be 170 μM and the V_{max} 26 units·mg^{-1}.

Inhibition studies were conducted by addition of dUDP, dUMP or PP_i to incubations where labelled dUTP was used as substrate. As dUDP is a reaction substrate for *Leishmania* dUTPase, it could act as a competitive inhibitor regarding dUTP hydrolysis [21]. Thus several concentrations of dUDP were tested for their inhibitory effect on dUTP hydrolysis at different dUTP concentrations. The assay was performed using 1 nM enzyme and substrate concentrations ranging between 1 and 100 μM. dUDP behaved as a competitive inhibitor, with a K_i of 15 μM. This value is identical to the value described previously for *E. coli* dUTPase [4].

Inhibition of dUTPase by the reaction products, dUMP and PP_i, was described formerly. In the case of the *E. coli* enzyme, both dUMP and PP_i act as weak substrate competitive inhibitors [4]. In order to analyse product inhibition of *Leishmania* dUTPase, the standard assay was carried out at different substrate concentrations in the presence of different concentrations of dUMP (5–100 μM) or PP_i (10–100 μM). dUMP behaved as a competitive inhibitor of the enzyme regarding dUTP hydrolysis, with a K_i value of 10 μM. However, there was no inhibition of *Leishmania* dUTPase by PP_i over the concentration range tested.

DISCUSSION

In a previous study [10] we showed that, as a result of screening by genetic complementation in the dUTPase-defective *E. coli* mutant BW286, an enzyme with dUTPase activity was characterized in *L. major*. However, in a database search no evident homology with eukaryotic dUTPases could be identified [10]. The best match was obtained with certain domains of dUTPase-dCTPase, an enzyme associated with the deoxynucleotide-synthesizing complex of the T4 phage of *E. coli* and which has the role of preventing incorporation of both dUTP and dCTP into a genome containing hydroxymethyl dCMP.

The deduced polypeptide had 269 amino acid residues and an inferred molecular mass of 31.42 kDa. Using a bacterial system we have overproduced and purified recombinant *L. major* dUTPase enzyme close to homogeneity and have determined that the protozoan enzyme exhibits particular properties that differentiate it from the other dUTPases. Functional analysis now shows that *Leishmania major* dUTPase is highly specific for dUTP, with little capacity for hydrolysing other NTPs. It is also active with dUDP. Moreover, apparently the active form of *Leishmania* dUTPase is a dimer, whereas most of the eukaryotic

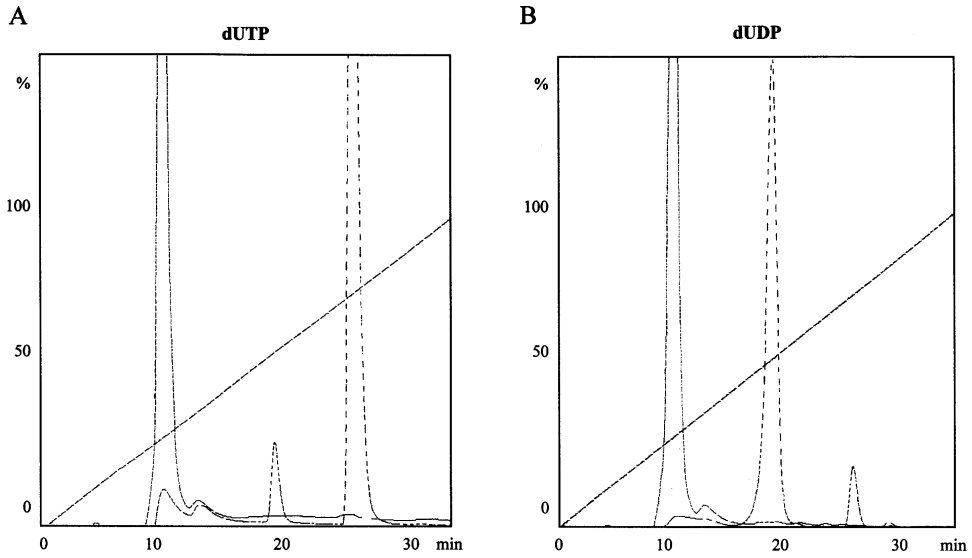

Figure 3 Effect of dUTPase on dUTP and dUDP

Samples containing 100 mM dUTP (**A**) or dUDP (**B**) were analysed by ion-exchange chromatography before (---) and after (———) incubation with *Leishmania* dUTPase. Retention times for the different nucleotides were 10.5 min for dUMP, 19.4 min for dUDP and 26.1 min for dUTP.

Table 4 Spectrophotometric determination of the P_i released during the course of the hydrolysis reaction by *Leishmania* dUTPase

The results are means ± S.E.M. of three experiments. Reactions 2, 3 and 6 correspond to the P_i released after treatment of the reaction products with 10 units of inorganic pyrophosphatase (PPase). Reactions 3 and 5 correspond to the P_i released in the absence of dUTPase. Reaction 6 corresponds to the P_i released in the absence of substrate and dUTPase.

Reaction	dUTPase (50 nM)	Substrate (1.5 mM)	PPase	P_i (mM)	PP_i (mM)	Hydrolysis (%)
1	+	dUTP	−	0.10 ± 0.01	—	—
2	+	dUTP	+	2.97 ± 0.05	1.48	99.00
3	−	dUTP	+	0.44 ± 0.02	—	—
4	+	dUDP	−	1.35 ± 0.03	—	90.38
5	−	dUDP	−	0.11 ± 0.01	—	—
6	+	−	+	0.39 ± 0.07	—	—

dUTPases described so far have been described as trimers [5–9] or, in the case of the herpes virus family, monomers [22,23].

The effect of magnesium on the *Leishmania* enzyme was analysed because most of the dUTPases characterized so far from different organisms are highly dependent on the presence of this metal. For instance, Mg^{2+} stimulates the activity of the human enzyme by 165% [24], the phage-induced dCTPase-dUTPase activity increases more than 3-fold in a Mg^{2+} concentration range of 0–20 mM [20] and the *Bacillus subtillis* and herpes simplex virus (HSV) dUTPases have an optimal activity in the presence of $MgCl_2$ of 50 mM [25]. By contrast, other dUTPases do not seem to have such strict requirements, as in the case of dUTPase from Yoshida sarcoma cells [19]. The absolute requirement of dUTPase for divalent metal ions suggests that it plays a role in catalysis, although studies on the tertiary structure of the human enzyme indicate that this requirement may be purely structural [7]. Accordingly, neither Ca^{2+} nor Na^+ would promote hydrolysis of dUTP by *Leishmania* dUTPase and only Mg^{2+} and Mn^{2+} are able to maintain this activity. It is possible that the greater bulk of the Ca^{2+} ion compared with that of the Mg^{2+} or Mn^{2+} ion distorts the binding of the substrate, avoiding its hydrolysis.

Another distinct characteristic is the observation that contrary to the majority of the dUTPases described so far, mouse [26], human [27], rat [28] and others, the activity of the purified recombinant enzyme was inhibited by NaF. A similar observation has been obtained in the case of the T4 phage dCTPase-dUTPase [20]. Likewise, similar to the T4 enzyme, the *Leishmania* enzyme was able to hydrolyse dUDP as efficiently as dUTP, the products being dUMP in both cases and P_i (dUDP) or PP_i (dUTP) respectively [20,29]. However, neither dCTP nor dCDP are substrates of the protozoan enzyme, which is a logical observation taking into account that dCMP is a normal component of DNA in trypanosomatids [30]. The limited homology with the T4 enzyme therefore does not imply that both enzymes act upon the same substrates. Rather, the *Leishmania* enzyme has apparently evolved to give a protein that can discriminate uracil from cytosine. Regarding product inhibition, dUMP acts as a relatively strong competitive inhibitor with a K_i of 10 μM whereas PP_i does not appear to inhibit the *Leishmania* enzyme. This value is approximately 10-fold lower than that described for other dUTPases [4].

So far, the exact role of the dUTPase in *Leishmania* is unknown, since the mechanisms by which dUTP arises have not been determined. The value of apparent K_m for dUTP was in a similar range to dUTPases described from other sources (0.2–12 μM), which is consistent with its role in maintaining very low dUTP concentrations in order to minimize uracil incorporation into DNA. Likewise, the turnover number was very similar to that obtained for the EIAV and HSV dUTPases [31,32]. It would be interesting to establish if dUTPase is required for the biosynthesis of all or only part of dUMP in *Leishmania*. In enteric bacteria, dUTP is an obligatory precursor for the biosynthesis of thymidylate. The deamination of dCTP followed by the hydrolysis of the dUTP formed appears to constitute the major route for dUMP formation (75%); meanwhile, 25% of this nucleotide is synthesized by dephosphorylation of the dUDP generated by the reduction of UDP [28]. The conversion of dUDP to dUMP is most probably also done via phosphorylation to dUTP. Nevertheless, in eukaryotic organisms and certain bacteria and bacteriophages, deamination occurs at the monophosphate level [29,33,34].

Since *Leishmania* dUTPase is significantly different from the human enzyme the observations of the present study suggest that the enzyme might be sufficiently different to be targeted selectively by specific inhibitors. Further studies regarding the three-dimensional structure of the enzyme will be useful in this respect. Recently, Bernier-Villamor et al. have obtained crystals of *Trypanosoma cruzi* dUTPase [35]. This enzyme displays 50.5% amino acid identity with the *Leishmania* counterpart and its characterization is currently being accomplished. Thus the presence of a nucleotidohydrolase with these characteristics may be a general issue in Trypanosomatids where, considering the kinetic parameters obtained in this study, it may have an important role in preventing incorporation of uracil into DNA. Likewise, the discovery that the modified base β-D-glucosyl-hydroxymethyluracil (J), apparently involved in gene-expression regulation, is a normal constituent of DNA in Kinetoplastida [30] emphasizes the importance of enzymes involved in uracil and thymine metabolism in these organisms.

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