Characterization of deoxyuridine 5′-triphosphate nucleotidohydrolase from *Trypanosoma cruzi*[1]

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Abstract **We report the cloning and kinetic characterization of *Trypanosoma cruzi* deoxyuridine 5′-triphosphate nucleotidohydrolase (dUTPase) whose coding sequence was isolated by genetic complementation in *Escherichia coli*. The deduced amino acid sequence was similar to *Leishmania major* dUTPase although it exhibits an amino acid insertion which is sensitive to protease inactivation. The catalytically active species of the enzyme is a dimer and a detailed kinetic characterization showed that it is highly specific for dUTP and dUDP. The general observation that dUTPases from the *Trypanosomatidae* differ in sequence, conformation and substrate specificity suggests that a different family of dUTPases exists in certain organisms, which may be exploited as drug targets against infectious diseases.** © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: dUTPase; dUDPase; Uracil; Nucleotide metabolism; Chagas' disease

1. Introduction

In most organisms, uracil must be methylated to thymine prior to its incorporation into DNA. Several enzymes play different roles in the synthesis and maintenance of thymine in DNA and some of them have been exploited as drug targets in different pathologies. Deoxyuridine 5′-triphosphate nucleotidohydrolase (dUTPase, EC 3.6.1.23) catalyzes the magnesium-dependent hydrolysis of dUTP to dUMP and pyrophosphate, providing the substrate for methylation of uracil by thymidylate synthase and preventing accidental incorporation of uracil into DNA by DNA-polymerase [1].

The gene encoding dUTPase (*dut*) is present in most organisms studied from viruses to higher eukaryotes. Deduced amino acid sequences show a significant similarity defined by 29–36% identity and five amino acid consensus motifs [2]. Monomeric and trimeric dUTPases have been described [2–4], that differ in the arrangement of these consensus motifs. For an efficient function, dUTPases have evolved to reach exquisite substrate recognition, and therefore the uracil ring, the sugar and even the triphosphate moieties are well distinguished. The structure of four trimeric dUTPases has been solved [4–8]. They all show the same general architecture formed by a β-barrel where the active sites are located in clefts between two subunits and a flexible arm from the third subunit crosses the molecule to act as a 'lid', which closes up when the substrate is bound.

In the present report we describe the isolation of a gene encoding a protein with dUTPase activity in *Trypanosoma cruzi*, the etiologic agent of Chagas' disease. The deduced amino acid sequence is similar to that reported for *Leishmania major*, differing both from the defined trimeric and monomeric family of dUTPases. Differences found in trypanosomatid dUTPases suggest that they belong to a different new family of dUTP pyrophosphatases that differ in structure and function to previously reported enzymes accomplishing the same function.

2. Materials and methods

2.1. Cloning of the TCDUT gene

A *T. cruzi* Y strain cDNA expression library was made in λ-ZAP Express™ c-DNA Gigapack® (Stratagene) and excision into the corresponding expression plasmids was performed assisted by helper λ phage.

Escherichia coli cells strain BW286 [Δ(*xth-pncA*), *dut-1*] were kindly supplied from Bernard Weiss (Johns Hopkins University, Baltimore, MD, USA) and transformed with the excised expression plasmids. Replicated growing of transformed cells, at 28°C and 37°C, was used. The colonies observed at 28°C served as a test for the background effect due to the frequent reversion in the phenotype of this strain [9].

Automated sequencing and oligonucleotide biosynthesis was performed at the Analytical Services of the Instituto de Parasitología y Biomedicina 'López Neyra'. The sequence of the *tcdut* gene is available in GenBank with the accession number U93211.

2.2. T. cruzi dUTPase overexpression and purification

Recombinant *T. cruzi* dUTPase was produced using the pET 11 system. Pellets of dUTPase overexpressing cells were resuspended in a solution consisting of buffer A (0.02 potassium phosphate, pH 6.5, 2 M dithiothreitol (DTT), 5 M $MgCl_2$) plus protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg ml^{-1} leupeptin, 1 mM benzamidine). All reagents were from Sigma, except IPTG and leupeptin, which were from Boehringer. The soluble crude extract, obtained by sonication, was directly loaded onto a hydroxyapatite chromatography column (Bio-Rad) at 1 ml min^{-1}. Elution was achieved with a linear gradient of potassium phosphate (20–200 mM).

Active fractions from hydroxyapatite were pooled, diluted 1:2 with buffer A and passed through a DEAE–cellulose chromatography col-

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[1] The nucleotide sequence reported here appears in DDBJ and GenBank nucleotide sequence databases under the accession number U93211.

Abbreviations: dUTPase, deoxyuridine 5′-triphosphate nucleotidohydrolase; DMT-dU, 5′-*O*-(4-4′-dimethoxytrityl)-2′-deoxyuridine

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umn (Whatman) pre-equilibrated with buffer A at 1 ml min^{-1}. Elution from this column was performed with NaCl in a linear gradient from 0 to 200 mM.

2.3. Activity measurements

Continuous hydrolysis of dUTP was followed spectrophotometrically taking advantage of the release of protons involved in the reaction [10]. Cocktails of buffer/indicator used were MES/bromocresol purple (375 μM/20 μM), pH 5.7–6.2, MOPS/bromothymol blue (625 μM/20 μM), pH 6.5–7.25, Bicine/cresol red (0.5–3 mM/25–50 μM), pH 7.5–8.5, CHES/alizarine yellow (2 mM/50 μM), pH 9.0–10.0. Other additives in the reaction mixtures included 1 mg ml^{-1} bovine serum albumin and 5 mM $MgCl_2$. pH indicators were all from Merck and the remaining reagents were from Sigma. Concentration of high pure nucleoside triphosphates (Pharmacia) or nucleoside diphosphates (Sigma) was evaluated spectrophotometrically prior to dilution in reaction buffer. Temperature for the measurements was set at 25°C.

Initial absorbance values were recorded by starting the reactions with a stopped-flow accessory (Hi-Tech Scientific) attached to a HP-8453 UV-Visible spectrophotometer. Wavelengths of maximal absorbance for the basic species of the pH indicator were used for the time course of the reaction. Data were collected and the resulting traces were analyzed using the Michaelis–Menten integrated rate equation with Microsoft Excel 97. For inhibition studies, K_i values were calculated as described [11]. 5′-*O*-(4-4′-dimethoxytrityl)-2′-deoxyuridine (DMT-dU) and 2′-deoxyuridine 5′-(α,β-imido) diphosphate were from Sigma and JenaBioScience respectively.

2.4. Crosslinking of dUTPase

The pure enzyme was incubated in 50 mM HEPES, pH 8.0, 0.1 mM EDTA, 10 mM $MgCl_2$, 0.5 mM DTT in the presence or absence of 100 μM disuccinimidyl suberate. The reaction was stopped at different time intervals through precipitation with equal volumes of 50% trichloroacetic acid and two consecutive washing steps with acetone. Finally, the pellets were dissolved in SDS–PAGE sample buffer and the sizes of the products generated in the reaction were analyzed in 12% polyacrylamide gels.

3. Results and discussion

3.1. Cloning of the TCDUT gene

In order to obtain the gene encoding dUTPase in *T. cruzi*, a screening technique based on the deficiency in dUTPase activity of the strain *E. coli* BW286 [9] was used. This approach had been successfully used in the cloning of the human and *Leishmania* dUTPase genes [12,13].

The analysis of the soluble crude extracts of positive clones by SDS–PAGE showed the presence of a 32 kDa protein overexpressed in two independent clones and a slightly higher molecular mass protein in a third clone. The cDNA sequence present in the first two clones was identical and corresponded to complete mature mRNAs of *T. cruzi*. The characteristic 'spliced leader' sequence, present in all mRNAs from trypanosomatids, was observed in the 5′-end [14] and the poly-A tail was present in the 3′-end. The first initiation codon was located 19 nucleotides downstream of the spliced leader and an open reading frame of 849 encoded a protein sequence of 283 amino acids. The calculated molecular mass and isoelectric point for the monomeric form were 32 065 Da and 5.16 respectively. The DNA sequence from the stop codon to the poly-A tail was different in size from one clone to the other (144 and 271 nucleotides respectively). The third clone contained a 5′-truncated version of the gene *TCDUT*, starting at the seventh coding triplet, and was expressed as a fusion protein together with the N-terminus of β-galatosidase. When the cDNA sequence was analyzed, no significant similarity could be established with the family of trimeric enzymes and, what is more, the consensus motifs were absent. In contrast *T. cruzi* dUTPase was highly similar to the reported sequence of the trypanosomatid *L. major* [13] and the eubacteria *Campylobacter jejuni* [15] (Fig. 1).

TCDUT also presents three motifs in common with an enzyme related in function, the dCTPase–dUTPase from phage T4 [13]. These motifs are conserved in the dCTPase–dUTPase from T2 and in dUTPases from *L. major* and *C. jejuni* (Fig. 1). The dCTPase has evolved to attain dUTPase activity and interestingly this enzyme has capacity for the hydrolysis of the diphosphates derivatives dCDP and dUDP [16]. In addition, an examination of the N-terminus reveals the existence of basic amino acids in positions +3 and +9 characteristic of mitochondrial targeting sequences in the Trypanosomatidae [17]. While distinct mitochondrial and nuclear

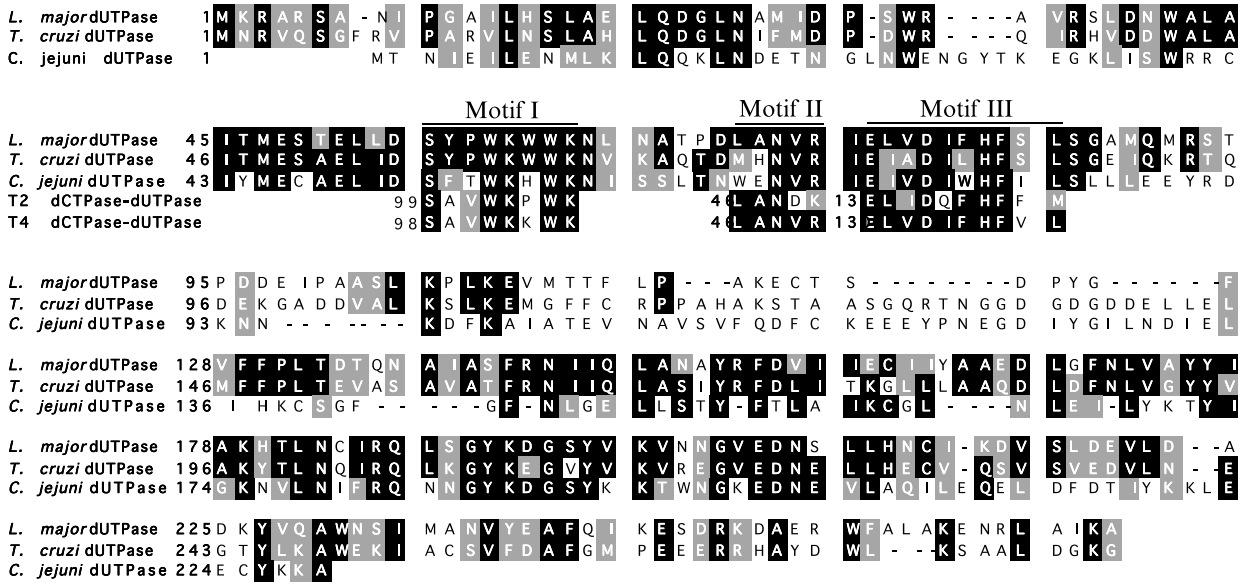

Fig. 1. Alignment of dUTPases from the trypanosomatids *T. cruzi* and *L. major* with the potential dUTPase gene from *C. jejuni*. Similarity with T-even phages dCTPase–dUTPases is also shown. The alignment was made using the default parameters of the program PILE UP from the GCG-package.

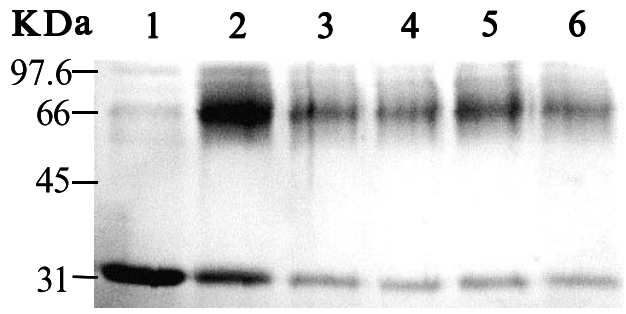

Fig. 2. Crosslinking of *T. cruzi* dUTPase. Lane 1: recombinant protein in the absence of DSS incubated 120 min at 37°C. Lanes 2–6: dUTPase incubated with 100 μM DSS during 5, 10, 30, 60 and 120 min respectively.

isoforms have been described to exist in human cells in agreement with the proposed role of dUTPase in DNA replication [18] the exact subcellular localization of dimeric dUTPases remains to be established. The availability of recombinant enzyme will allow for antibody production and detailed intracellular localization studies.

3.2. Native molecular mass

The molecular mass of the native protein was investigated in pure recombinant protein by gel filtration in Superdex 75 and was determined to be 62 445 Da in phosphate buffer both in the presence and absence of 100 mM of magnesium, suggesting that *T. cruzi* dUTPase is a stable dimer.

Subunit arrangement was also studied by using the crosslinking agent DSS (suberic acid bis (*N*-hydroxy-succinimide ester)). Analysis by SDS–PAGE showed the presence of a 64 kDa species which is absent in the lane corresponding to untreated enzyme (Fig. 2) and again agrees with a dimeric arrangement in the active protein unit. This result also agrees with the fact that two molecules of protein per asymmetric unit were described in crystals of this protein [19].

In the process of purification of recombinant *T. cruzi* dUTPase the protein is cleaved into two peptides of similar size when PMSF is omitted in the buffers. The size of the peptides generated, 15 and 17 kDa as estimated by SDS–PAGE, suggests that *T. cruzi* dUTPase is specifically recognized and cleaved in the amino acid insertion not present in the sequence of *L. major* (Fig. 1). The cleaved protein showed a dimeric arrangement when analyzed by gel filtration and was still active (results not shown), suggesting that no dramatic changes are originated in the overall structure after proteolytic degradation.

3.3. Kinetic characterization and inhibition

Although the analysis of the progress curve obtained with the Michaelis–Menten integrated rate enables calculation of K_m from a single curve, distinct values of K_m were obtained when the initial concentration of dUTP varied in the assay. The apparent K_m increased at higher concentrations of substrate, suggesting a process of product inhibition similar to what has been described for the *Leishmania* enzyme [20]. The integrated rate equation becomes more complex in this case [21] and the estimation of K_ms and inhibition constant of the product (K_{mp}) is performed by replotting K_m apparent against initial dUTP concentrations. These calculations gave a value for K_m of 0.534 μM. On the other hand, V_{max} remained constant at different dUTP concentrations and permitted an estimation of k_{cat} of 2.8 s^{-1}, assuming two active sites per dimer of dUTPase. The study of the kinetic behavior of the enzyme demonstrated its equivalence in function previously reported [11,22]. While the low K_m values described so far (0.1–12 μM) would ensure very low dUTP concentrations in the cell, the value of k_{cat}, in the range of 1.5–25 s^{-1}, is relatively low in comparison with other enzyme families (Table 1). *T. cruzi* dUTPase was tested for capacity of hydrolysis of the nucleosides triphosphate common in the cell (UTP, dTTP, dCTP, dGTP and dATP). No appreciable hydrolysis was observed in conditions where dUTP is fully hydrolyzed and only in the case of dTTP a slow reaction appears to occur. The determination of the kinetic constants for this pseudo-substrate required much higher concentrations of both enzyme and substrate, and the application of the integrated rate equation was impracticable due to inability to reach a situation of substrate saturation. Alternatively, a differential method for analysis of the progress curve was applied [23]. This method permitted an estimation of the kinetic parameters, showing a specificity constant K_m/k_{cat} 10^3-fold lower than dUTP (Table 1).

A novel feature of trypanosomatid dUTPases is the observation that dUDP is an efficient substrate. Most dUTPases studied so far do not hydrolyze dUDP, which rather acts as a competitive inhibitor [5,8,10]. The kinetic parameters of this substrate were studied in detail. Again, an important product inhibition was observed and the kinetic parameters were investigated by reacting enzyme with different initial concentrations of substrate. The k_{cat} and K_m values obtained for dUDP were three- and six-fold higher respectively, than for the triphosphate derivative (Table 1).

Finally, the inhibition by DMT-dU, has been analyzed at concentrations from 10 to 1500 μM. While this compound is an efficient inhibitor of *E. coli* dUTPase, the K_i value obtained for the *T. cruzi* enzyme was greater than 2500 μM indicating again, as for the *Leishmania* enzyme [11], possible structural differences and the possibility of a specific inhibition of dimeric enzymes. On the other hand, α-β-imido-dUDP proved to be an effective inhibitor of the enzyme with a K_i value of

Table 1
Michaelis–Menten, specificity and inhibition constants of viral, bacterial and trypanosomatid dUTPases towards different nucleotides

Nucleotide	*T. cruzi*	*L. major*	*E. coli*	EIAV	HSV-1	MMTV
dUTP: K_m (k_{cat}/K_m)	0.5 (5.2×10^6)	2.1 (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)	>800 (2.5×10^3)	1514 (5.5×10^3)	>20000 (34)	260 (<2000)	400 (1000)	nd (2000)
dCTP: K_m (k_{cat}/K_m)	nd	>2500 (5.0×10^3)	4000 (<100)	3000 (1000)	1000 (2000)	nd
UTP: K_m (k_{cat}/K_m)	nd	>2500 (2.0×10^3)	2500 (<100)	nd	1000 (200)	nd
dUDP: K_m (k_{cat}/K_m)	6.2 (1.710^6)	62.7 (1×10^6)	K_i = 15	K_i = 3.6	K_i = 17	nd
dUMP: K_{ip}	18.4	13.1	1500	130	170	nd

Units for K_m and K_i are μM and for k_{cat}/K_m M^{-1} s^{-1}. nd, not determined.

0.24 μM, in agreement with the observation that the enzyme binds effectively nucleoside diphosphates.

In summary, no sequence similarity with other dUTPases, dimeric conformation of the active protein and efficient hydrolysis of dUDP are all features common to trypanosomatid dUTPases which appear to form a new family of dUTPases with distinct structural and functional characteristics. Crystals of *T. cruzi* dUTPase have been obtained [19]. The determination of the three dimensional structure of this protein will help to define specific differences that may be exploitable in the design of inhibitors useful in the development of new drugs for Chagas' disease therapy.

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References

[1] Warner, H.R., Duncan, B.K., Garret, C. and Neuhard, J. (1981) J. Bacteriol. 145, 687–695.
[2] McGeoch, D.J. (1990) Nucleic Acids Res. 18, 4105–4110.
[3] Caradonna, S.J. and Adamkiewicz, D.M. (1984) J. Biol. Chem. 259, 5459–5464.
[4] Cedergren-Zeppezauer, E.S., Larsson, G., Nyman, P.O., Dauter, Z. and Wilson, K.S. (1992) Nature 335, 740–743.
[5] Larsson, G., Svensson, L.A. and Nyman, P.O. (1996) Nat. Struct. Biol. 3, 532–538.
[6] Mol, C.D., Harris, J.M., McIntosh, E.M. and Tainer, J.A. (1996) Structure 4, 1077–1092.
[7] Prasad, G.S., Stura, E.A., McRee, D.E., Laco, G.S., Hasselkus-Light, C., Elder, J.H. and Stout, C.D. (1996) Protein Sci. 5, 2429–2437.
[8] Dauter, Z., Persson, R., Rosengren, A.M., Nyman, P.O., Wilson, K.S. and Cedergren-Zeppezauer, E.S. (1999) J. Mol. Biol. 285, 655–673.
[9] Taylor, A.F. and Weiss, B. (1982) J. Bacteriol. 151, 351–357.
[10] Larsson, G., Nyman, P.O. and Kvassman, J.O. (1996) J. Biol. Chem. 271, 24010–24016.
[11] Hidalgo-Zarco, F., Camacho, A.G., Bernier-Villamor, V., Nord, J., Ruiz-Pérez, L.M. and González-Pacanowska, D. (2001) Protein Sci. 10, 1426–1433.
[12] McIntosh, E.M., Ager, D.D., Gadsden, M.H. and Haynes, R.H. (1992) Proc. Natl. Acad. Sci. USA 89, 8020–8024.
[13] Camacho, A., Arrebola, R., Peña-Diaz, J., Ruiz-Pérez, L.M. and González-Pacanowska, D. (1997) Biochem. J. 325, 441–447.
[14] McCarthy-Burke, C., Taylor, Z.A. and Buck, G.A. (1989) Gene 82, 177–189.
[15] Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D., Chillingworth, T., Davies, R.M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A.V., Moule, S., Pallen, M.J., Penn, C.W., Quail, M.A., Rajandream, M.A., Rutherford, K.M., Van Vliet, A.H., Whitehead, S. and Barrell, B.G. (2000) Nature 403, 665–668.
[16] Warner, H.R. and Barnes, J.E. (1966) Proc. Natl. Acad. Sci. USA 56, 1233–1240.
[17] Häusler, T., Stierhof, Y.D., Blattner, J. and Clayton, C. (1997) Eur. J. Cell Biol. 73, 240–251.
[18] Ladner, R.D. and Caradonna, S.J. (1997) J. Biol. Chem. 272, 19072–19080.
[19] Bernier-Villamor, V., Camacho, A., González-Pacanowska, D., Cedergren-Zeppezauer, E., Antson, A. and Wilson, K.S. (1999) Acta Cryst. D55, 528–530.
[20] Camacho, A., Hidalgo-Zarco, F., Bernier-Villamor, V., Ruiz-Perez, L.M. and Gonzalez-Pacanowska, D. (2000) Biochem. J. 346, 163–168.
[21] Segel, I.H. (1975) Enzyme Kinetics: Behaviour and Analysis of Rapid Equilibrium and Steady-State Enzyme-Systems, Wiley Interscience, John Wiley and Sons.
[22] Nord, J., Larsson, G., Kvassman, J.O., Rosengren, A.M. and Nyman, P.O. (1997) FEBS Lett. 414, 271–274.
[23] Koerber, S.C. and Fink, A.L. (1987) Anal. Biochem. 165, 75–87.