The tyrosine aminotransferase from *Trypanosoma rangeli*: sequence and genomic characterization

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Abstract

The complete sequence and genomic characterization of the tyrosine aminotransferase (TAT) gene from *Trypanosoma rangeli* is reported. The gene was found to be organized in a tandem multicopy gene array. A homologous mRNA species (2.5 kb) was identified in the epimastigote form of the parasite. From the deduced amino acid sequence, the gene encodes a protein of 420 amino acids with a predicted molecular mass of 46.4 kDa and a theoretical p*I* of 6.23. A high sequence identity was found with the *Trypanosoma cruzi*, human and rat enzymes. All the essential residues for TAT enzymatic activity are conserved, as well as a pyridoxal-phosphate attachment site typical of class-I aminotransferases. The recombinant enzyme was recognized by a monoclonal antibody against the *T. cruzi* enzyme. Additionally, the recombinant protein showed enzymatic activity when incubated with L-tyrosine and 2-oxoglutaric acid as substrates.

Keywords: Tyrosine aminotransferase; Tandemly repeated gene; *Trypanosoma rangeli*; *Trypanosoma cruzi*

1. Introduction

Tyrosine aminotransferase (TAT) activity, involved in the initial step of tyrosine catabolism, has been described in several organisms (mammals, *Crithidia fasciculata*, *Tetrahymena pyriformis*, etc.), but few TATs accounting for that activity have been fully characterized. TAT activity was found in *Trypanosoma cruzi* (Tc) [1], the causative agent of Chagas' disease, and the enzyme purified [2] and the gene sequenced [3]. In spite of the high similarity to the human and mouse counterparts (77%), several differences in enzymatic properties were seen [2]. *Trypanosoma rangeli* (Tr) is coendemic with Tc in many areas, and they share a similar morphology, common epitopes (producing serological cross-reactions) and one of the insect vectors (*Rhodnius prolixus*). Previous results demonstrated TAT activity in Tr whole homogenate (unpublished results) and also suggested the presence of a TAT gene. A heterologous TAT probe from Tc hybridized to Tr chromosomes larger than 1.7 megabases in 16 different Tr stocks [4]. Here we describe the sequence and genomic characterization of Tr TAT, and report that the recombinant protein is enzymatically active.

2. Materials and methods

2.1. Growth of parasites

Tr epimastigotes of LDG strain and clone D1-D2-Duarte strain N2378 were grown in BHT medium (brain–heart–tryptose) supplemented with 10% fetal calf serum (Sigma) at 28°C.

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2.2. Southern and Northern blot analysis

Total DNA was digested overnight with several enzymes, or for variable periods of time with *Hin*dIII and *Pst*I, separated on a 1% agarose gel, capillary-transferred to Biodyne nylon membranes and baked. Total RNA was purified from 10^9 parasites using the acid phenol method with Trizol (Gibco BRL) following the manufacturer's instructions. A 1.5% agarose gel was prepared in 20 mM MOPS pH 7, 5 mM sodium acetate, 1 mM EDTA, 0.66 M formaldehyde. Twenty μg of total RNA plus ethidium bromide were loaded per well. The front was run two thirds of the length at 1 V cm^{-1}. The separated RNA was capillary-transferred in 10×SSC to a nylon membrane and cross-linked with UV light [5]. [α-^{32}P]dCTP and Ambion DECAprime II™ Random Priming DNA Labeling kit were used to label the probes.

2.3. Cloning and sequencing

Digested genomic DNA and fragments of the gene were purified from agarose gels using DEAE membranes [6]. After restriction mapping, several fragments were subcloned into pUC vector. DNA sequencing was performed in a Perkin-Elmer ABI 377 equipment (Servicio de Secuenciación, ANLIS, Argentina) using a BigDye Terminator Cycle Sequencing kit (Perkin-Elmer, USA).

2.4. Expression and purification of the recombinant protein

A fragment containing the entire coding sequence of the gene was cloned into pQE 32 vector digested with *Bam*HI and *Pst*I (Qiagen) to produce a fusion protein carrying a tag of six histidines at the N-terminal end. An overnight culture was diluted 10 times, grown until OD_{600} 0.5–0.7, induced with 1 mM IPTG for 3 h and centrifuged. The bacterial pellet was resuspended in 5 ml of lysis buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 10 mM imidazole). Cells were lysed on ice by sonication and centrifuged at 10 000×*g* at 4°C for 20 min. Five ml of the supernatant was loaded onto 1 ml of the Ni^{2+}-nitrilo triacetic acid resin affinity column (Qiagen), previously equilibrated with lysis buffer. The column was then washed with 10 ml of washing buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 20 mM imidazole) and eluted with elution buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 250 mM imidazole). The purified native recombinant protein was quantitated by Bradford's method [7].

2.5. SDS–PAGE and Western blot

Sample proteins were boiled in the presence of SDS and β-mercaptoethanol as reducing agent, separated in a 10% acrylamide gel and transferred to nitrocellulose. Half of the blot was stained with amido black. After blocking with skimmed milk in phosphate-buffered saline, the other half was incubated for 1 h at room temperature with monoclonal antibody 2C5D6, generated against Tc TAT (A.M. Ruiz, G.A. García, unpublished results). Three washes with phosphate-buffered saline preceded incubation with anti-mouse IgG conjugated to horseradish peroxidase (Jackson). After a washing step, the blot was developed with 4-chloro-1-naphthol (Sigma).

2.6. Enzymatic activity of recombinant bacteria lysates

Induced recombinant bacteria were harvested by centrifugation and resuspended (0.5 g ml^{-1}) in 25 mM phosphate buffer pH 7.8, 120 mM KCl, 1 mM dithiothreitol (DTT), 2.5 mM 2-oxoglutaric acid, 0.2 mM pyridoxal 5-phosphate and 1 mM phenyl methyl sulfonyl fluoride. After sonication in a Branson sonifier (six pulses, 30 s each, maximum power) and centrifugation (30 min, 16 000×*g*), enzymatic activity was tested in the supernatant. Ten–50 μl was incubated in the reaction mixture (100 mM HEPES buffer, pH 7.0, 1.2 mM EDTA, 0.6 mM DTT, 0.1 mM pyridoxal 5-phosphate, 28 mM 2-oxoglutaric acid and 2 mM L-tyrosine) for 30 min at 37°C. The reaction was stopped by adding potassium hydroxide (final concentration 0.94 M) and incubated 20 min at room temperature. Five-fold dilutions of samples were measured for absorbance at 331 nm. One unit was defined as the amount of enzyme catalyzing the formation of 1 μmol of *p*-hydroxyphenylpyruvic acid (measured as *p*-hydroxybenzaldehyde, molar absorbance 19900 M^{-1} cm^{-1} at 331 nm) per min at 37°C. The presented results are the mean of three independent experiments.

3. Results and discussion

3.1. Isolation, total sequencing and genomic organization of the gene

Having demonstrated TAT activity in Tr whole homogenate (data not shown), we undertook to search for the gene. Genomic DNA (LDG strain), which had previously been digested with the indicated restriction enzymes, was separated by gel electrophoresis, transferred to a nylon membrane and probed with a labeled DNA fragment comprising the last 900 bp of the coding region of Tc TAT gene [3]. A band of approximately 2 kb was detected in the lane corresponding to *Hin*dIII-digested DNA in a Southern blot (not shown). Hence a similar digestion was separated on a 1% agarose gel, and DNA from the 2.3–1.7-kb region was purified. Cloning into pUC and screening of the resulting colonies with the same probe resulted in three positive colonies. Following confirmation by DNA sequencing, the inserts were mapped by restriction analysis. Subcloning of selected restriction fragments allowed the complete sequence of the gene to be determined (GenBank accession number AF165323).

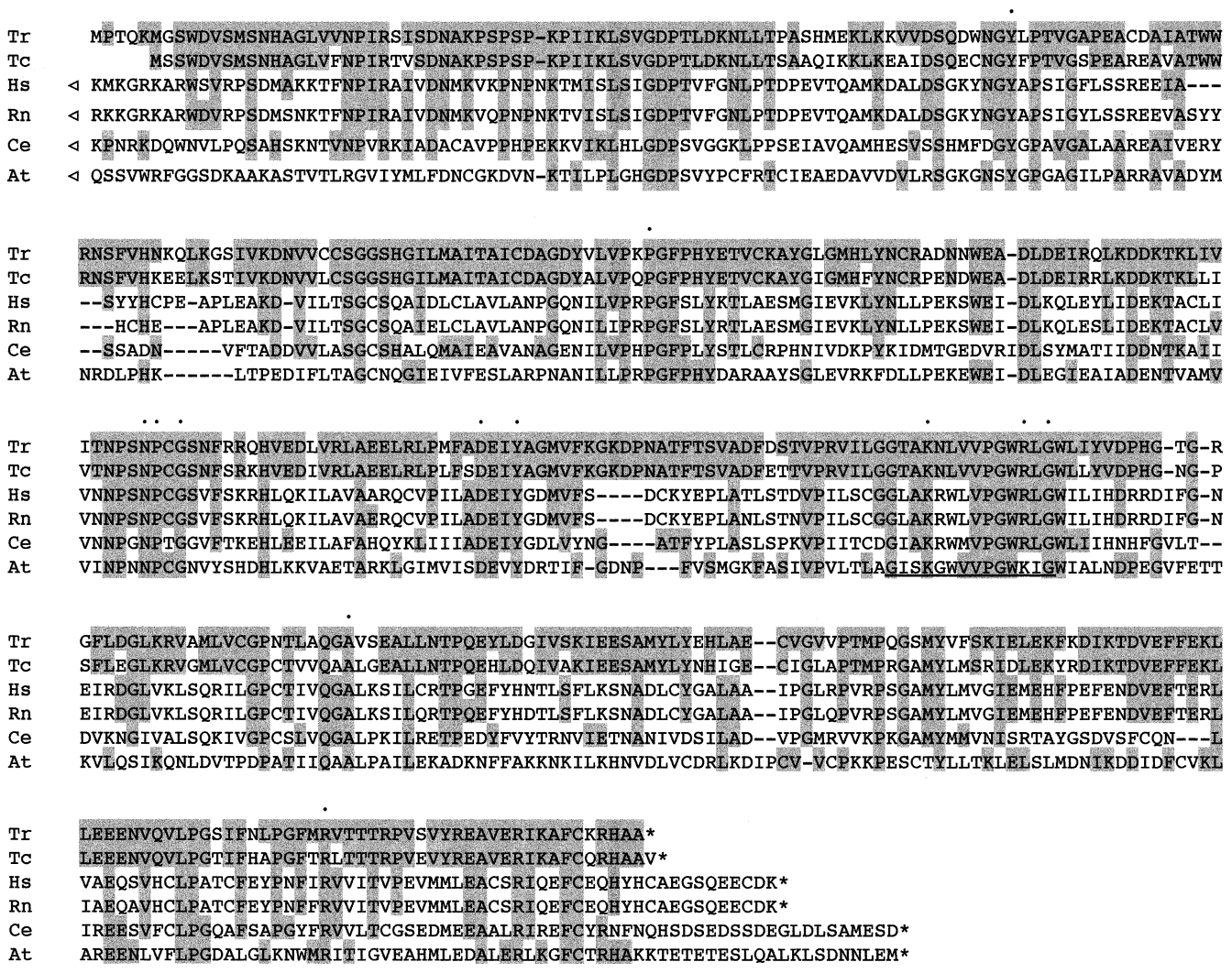

Fig. 1. Comparison of Tr TAT with those from several species: Tc (Tc, L00673), human (Hs, P17735), *Rattus norvegicus* (Rn, P04694), *Caenorhabditis elegans* (Ce, Z81081) and *Arabidopsis thaliana* (At, AAD21706). Identical residues are shaded. The triangles represent a variable length of residues before the first potential amino acid from Tr TAT: 32 (Hs and Rn), 35 (Ce) and 28 (At). Gaps, indicated by dashes, were introduced to improve the alignment. Bullets mark the essential residues for enzymatic activity. The pyridoxal-phosphate attachment site is underlined.

The nucleotide sequence shows three putative ATGs, the first one being closer to the Kozak consensus sequence determined for kinetoplastids in the −3 and +5 positions [8]. The gene spans over 1260 nucleotides, from the first putative ATG to the stop codon, having the capacity to encode a 420-amino acids protein. The theoretical molecular mass is 46.4 kDa, and the isoelectric point 6.23 (ExPASy server).

A search for motifs using PROSITE [9] disclosed a putative N-glycosylation site and several phosphorylation sites. A pyridoxal-phosphate attachment site typical of class-I aminotransferases (PS00105) was also detected (Fig. 1). Comparing the putative coding sequence to protein databases using the BlastP program unveiled several TATs. The best identity was found with Tc (80%), followed by rat (37%) and human (36%) (Fig. 1).

Partial digestion of total DNA with restriction enzymes having a unique target site on a specific genomic fragment typically generates a ladder when multiple copies of the fragment are organized in a tandem array. Indeed, this was the result obtained after partial digestion of genomic Tr DNA using *Hin*dIII and *Pst*I, being 2 kb the length of the repeat unit (Fig. 2). This genomic organization parallels the situation found in Tc [3].

3.2. Expression and purification of the recombinant protein

The expression of the protein by the parasite was primarily evaluated in a Northern experiment. When Tr epimastigote total RNA was tested for the presence of specific messenger sequences, using the codifying 1–1240-bp DNA fragment of the cloned TAT gene as a probe, a strong band of 2.5 kb and two weak bands of 4.6 and 5.2 kb were detected (Fig. 3). The additional bands could represent transient processing forms.

The gene was subcloned into pQE and expressed. The

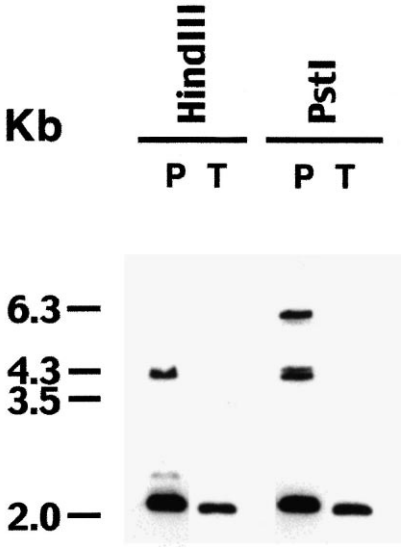

Fig. 2. Southern blot analysis of partially (P) and totally (T) digested genomic Tr DNA hybridized with a labeled fragment spanning Tr TAT coding region. The characteristic ladder of tandemly arranged genes is seen in lanes containing the *Hin*dIII and *Pst*I partial cleavages.

soluble recombinant protein, whose electrophoretic migration corresponded well with the theoretical expected value, was purified (Fig. 4A). Recognition of the native and recombinant proteins by the monoclonal antibody 2C5D6, which specifically inhibits Tc TAT, confirmed their identity (Fig. 4B). This result, together with the recognition of specific mRNA in the Northern experiment, and the compliance of Tr TAT gene with the codon usage of trypanosomatidae [10], strongly indicates that the gene we have cloned is actually expressed into protein in the parasite.

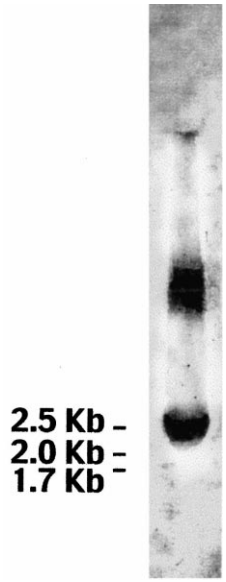

Fig. 3. Northern blot of total Tr RNA hybridized with a labeled probe comprising the cloned Tr TAT gene. Ethidium bromide-stained ribosomal RNA bands were used as molecular mass standards.

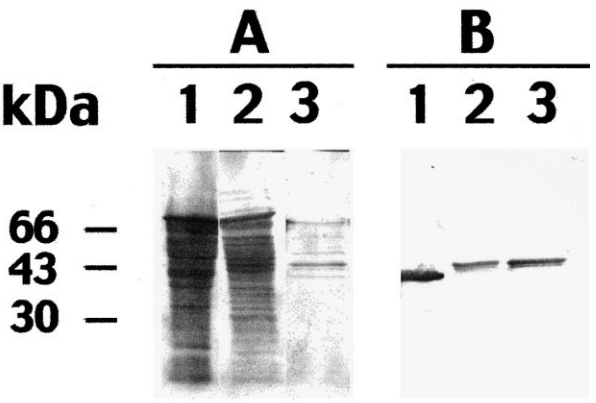

Fig. 4. Expression and recognition of the enzyme. SDS–PAGE and Western blot of Tr whole homogenate (clone D1-D2-Duarte strain N2378) (lane 1), lysate supernatant of bacteria expressing Tr TAT (lane 2) and column-purified recombinant Tr TAT (lane 3). A: Coomassie blue-stained gel. B: Western blot using monoclonal antibody 2C5D6.

3.3. Enzymatic activity of the recombinant protein

Two preliminary elements strongly suggested this gene could encode an active enzyme: (1) the identification of the cofactor binding site, in the correct position as compared with other TAT enzymes with demonstrated activity; and (2) the presence of the 12 essential residues for TAT activity (Fig. 1). In order to experimentally confirm this possibility, we assayed TAT activity in bacteria transformed with the cloned gene. TAT specific activity measured in the induced bacteria lysate (0.284 U mg^{-1}) was 10-fold higher than the control (non-recombinant bacteria, 0.022 U mg^{-1}).

It should be noted that this confirmatory result does not preclude the existence of other enzymes displaying the same function. In fact, the TAT activity detected in *C. fasciculata* [11] was afterwards shown to be performed by an aspartate aminotransferase-like enzyme [12].

Based on the broad specificity displayed by some aminotransferases, further work involving the full characterization of this enzyme will establish the level of functional similarity to the one from its close relative, Tc.

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