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Characterization of the M32 metallocarboxypeptidase of *Trypanosoma brucei*: Differences and similarities with its orthologue in *Trypanosoma cruzi*

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ABSTRACT

Metallocarboxypeptidases (MCP) of the M32 family of peptidases have been identified in a number of prokaryotic organisms but they are absent from eukaryotic genomes with the remarkable exception of those of trypanosomatids. The genome of *Trypanosoma brucei*, the causative agent of Sleeping Sickness, encodes one such MCP which displays 72% identity to the characterized *Tc*MCP-1 from *Trypanosoma cruzi*. As its orthologue, *Tc*MCP-1, *Trypanosoma brucei* MCP is a cytosolic enzyme expressed in both major stages of the parasite. Purified recombinant *Tb*MCP-1 exhibits a significant hydrolytic activity against the carboxypeptidase B substrate FA (furylacryloil)-Ala-Lys at pH 7.0–7.8 resembling the *T. cruzi* enzyme. Several divalent cations had little effect on *Tb*MCP-1 activity but increasing amounts of Co^{2+} inhibited the enzyme. Despite having similar tertiary structure, both protozoan MCPs display different substrate specificity with respect to P1 position. Thus, *Tc*MCP-1 enzyme cleaved Abz-FVK-(Dnp)-OH substrate (where Abz: *o*-aminobenzoic acid and Dnp: 2,4-dinitrophenyl) whereas *Tb*MCP-1 had no activity on this substrate. Comparative homology models and sequence alignments using *Tc*MCP-1 as a template led us to map several residues that could explain this difference. To verify this hypothesis, site-directed mutagenesis was undertaken replacing the *Tb*MCP-1 residues by those present in *Tc*MCP-1. We found that the substitution A414 M led *Tb*MCP-1 to gain activity on Abz-FVK-(Dnp)-OH, thus showing that this residue is involved in specificity determination, probably being part of the S1 sub-site. Moreover, the activity of both protozoan MCPs was explored on two vasoactive compounds such as bradykinin and angiotensin I resulting in two different hydrolysis patterns.

1. Introduction

Carboxypeptidases are enzymes that cleave the C-terminal amino acid residue from peptides and proteins. This hydrolysis step may result in substrate activation or degradation [1]. Regardless the outcome, this activity belongs to two major catalytic classes of proteases, namely metallocarboxypeptidases (MCPs) and serine carboxypeptidases [1]. MCPs are ubiquitous in nature and typically have a single zinc ion bound to the active site. Among carboxypeptidases, the family M14 is the most extensively studied group. This family includes several enzymes which participate in diverse processes such as blood coagulation and fibrinolysis, inflammation, innate immunity response, food digestion and pro-hormone and neuropeptide processing [2].

Previous studies on *Thermus aquaticus*, an extremely thermophilic bacterium, led to the identification of a new zinc-dependent MCP [3]. Contrary to archetypical M14 peptidases, *Thermus aquaticus* carboxypeptidase (*Taq*CP) was distinct in both size and sequence. *Taq*CP contained a HEXXH signature which is a conserved sequence found in the active site of neutral metalloendopeptidases, but not described previously in a carboxypeptidase. These facts led to the placement of *Taq*CP in the newly formed M32 family of MCPs [4]. Later studies performed on other members of this family also confirmed that this group of enzymes had also strong topological similarity with archaeal, bacterial and mammalian endopeptidases including angiotensin I-converting enzyme (ACE), neurolysin and thimet oligopeptidase [4,5].

Abbreviations: BSA, bovine serum albumin; BSF, bloodstream form; CPB, carboxypeptidase B; E64, L-transepoxysuccinyl-leucylamido-[4-guanidino] butane; FRET, Fluorescence Energy Resonance Transfer; GEMSA, guanidinoethylmercaptosuccinic acid; IMAC-Ni^{2+}, immobilized metal affinity chromatography using Ni^{2+}; MOPS, 3-(N-morpholino) propanesulfonic acid; NP40, nonyl phenoxypolyethoxylethanol; Plummer's inhibitor, DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid; PMSF, phenylmethylsulfonyl fluoride; PCF, procyclic form; TD Buffer, trypanosome dilution buffer.

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But not only the structural similarity to endopeptidases as well as their broad substrate specificity were the new features of these peptidases; their phylogenetic distribution was also quite peculiar. Sequence alignments have identified M32 family members in species from a very limited phylogenetic range. These include bacteria, archaea, some green algae and trypanosomatids, but not metazoa, suggesting that the genes have been acquired by horizontal gene transfer or retained for some special function that is no longer essential for higher organisms [6].

The Trypanosomatidae family includes several vector-borne protist parasites, causing human and veterinary diseases. The human parasites include a number of species in the genera *Leishmania* and *Trypanosoma*. In *Trypanosoma*, the two major human parasites are *T. cruzi*, the causative agent of Chagas' disease, and *T. brucei*, the causative agent of the Human African trypanosomiasis (HAT) or Sleeping Sickness. Both parasites are responsible for over 60,000 deaths per year in Latin America and Sub-Saharan Africa, respectively. For these infections no vaccines are available, and their treatment is dependent on a small number of drugs that have limited efficacy and can cause severe side effects [7]. These limitations make urgent the need to develop new drugs, more efficient, less toxic and more affordable [8]. In this context the study of peptidases has acquired considerable importance since several works have shown that many parasites deploy proteinases to accomplish both "housekeeping" tasks common to many eukaryotes as well as functions highly specific to the parasite life style. Host cell invasion and egress, encystation, excystation, catabolism of host proteins, differentiation, cell cycle progression, cytoadherence, and both stimulation and evasion of host immune responses are some processes in which this group of enzymes has been shown to be involved [9]. Thus, the possibility of developing selective inhibitors of key proteases of pathogenic parasites is currently explored as a novel chemotherapeutic strategy [9].

Herein we focus on *T. brucei*, an extracellular parasite, transmitted by the bite of the tsetse fly (*Glossina* spp.). The disease caused by this protozoan progresses from non-specific signs to typical symptoms such as wake–sleep cycle alteration and neurological dysfunction, leading to death in untreated patients [10]. In this work we report the cloning, purification and biochemical characterization of an MCP belonging to the M32 family (*Tb*MCP-1) from *T. brucei* Lister 427. These results add to our knowledge of the peptidases present in *T. brucei* and may offer a new target for the development of a rational chemotherapy against trypanosomiasis.

2. Materials and methods

2.1. Materials

Peptide substrates were purchased from Sigma–Aldrich and Bachem Bioscience, except for those with the structure Abz-XXK(Dnp)-OH [Abz: *o*-aminobenzoic acid; K(Dnp): N′,2,4-dinitrophenyllysine, and X different amino acid residues], which were synthesized as described in Ref. [11]. All other reagents were purchased from Sigma unless otherwise stated.

2.2. Trypanosome strains and culture conditions

The *T. brucei* bloodstream form (BSF) Lister 427 "single marker" trypanosome cell line (T7RNAPol TetR NEO) and the procyclic form (PCF) 29–13 cell line (T7RNAPol NEO TetR HYG) [12] were gifts from G.A.M. Cross (Rockefeller University). BSF cells were maintained in HMI-9 medium supplemented with 20% heat-inactivated fetal calf serum [13]. *T. brucei* PCF were grown at 28 °C in medium SDM-79 [14] supplemented with haemin (7.5 mg/l) and 10% heat-inactivated fetal-calf serum.

2.3. Genomic DNA purification and molecular cloning

In order to clone the MCP gene from *T. brucei* Lister 427, two synthetic oligonucleotide primers were designed: ATG (5′-GGATCCatgaagggcatacaaagagctcg-3′) and STOP (5′-GAATTCtcagttggcatcgtcacggtag-3′). PCR amplification was carried out using genomic DNA as template. PCR conditions were as follows: initial denaturation (5 min at 94 °C), denaturation (1 min at 94 °C), annealing (45 s at 62 °C) and elongation (90 s at 72 °C) followed by a final extension step (10 min at 72 °C). The PCR products were purified from a 1% agarose gel, using the QiaQuick protocol (Qiagen) and cloned into pGEM-T Easy vector (Promega). Sequencing of the products was performed using an ABI 377 DNA sequencer (PerkinElmer).

Site-directed mutations were introduced into the *Tb*MCP-1 gene cloned in the plasmid pGEX 2T, using the QuickChange™ site-directed mutagenesis kit (Stratagene).

DNA was obtained from PCF of *T. brucei* by using the Proteinase K/phenol/chloroform method [15].

2.4. Expression and purification of TbMCP-1 fused to glutathione S-transferase (GST)

The *T. brucei* MCP-1 gene (*Tb*MCP-1) was excised as BamHI/EcoRI fragment from pGEM-T Easy plasmid (Promega), gel purified and subcloned into the BamHI and EcoRI sites of the pGEX 2T expression vector. The resulting construct presented a GST tag at the N-terminus with a thrombin cleavage site. The construct was transformed into *E. coli* BL21 Codon Plus (DE3) cells. GST fusion protein was expressed by induction of exponential phase cultures (A_{600} = 0.6) with 0.5 mM IPTG for 12 h at 18 °C with vigorous (250 rpm) shaking. Bacteria were harvested by centrifugation at 5000 × *g* for 30 min at 4 °C, resuspended in 50 mM Tris–HCl pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 1 mM PMSF and 1 mg/ml lysozyme, and centrifuged at 12,000 × *g* for 30 min at 4 °C to obtain the bacterial crude extract.

The recombinant *Tb*MCP-1 fusion protein was purified using a glutathione-agarose resin (GE) equilibrated with 50 mM Tris–HCl pH 7.6 containing 150 mM NaCl. The column was washed with 10 column vol. of the equilibration buffer and the sample was eluted with 50 mM Tris–HCl pH 7.6, 150 mM NaCl containing 10 mM reduced glutathione. Samples were desalted using a PD-10 column (GE) according to the manufacturer's specifications. Protein purity was evaluated on a Coomassie Blue-stained SDS 10% polyacrylamide gel. A 6xHis-tagged version of *Tb*MCP-1 was constructed as in the case of *Tc*MCP-1 [6], and the recombinant enzyme was purified by IMAC-Ni^{2+}. This recombinant *Tb*MCP-1 was very unstable, but could be used to exclude the influence of the GST tag in enzyme dimerization and kinetics. Recombinant *Tc*MCP-1 from *T. cruzi* was purified as described in [5].

2.5. Enzyme assays

Routinely, recombinant *Tb*MCP-1 activity was assayed using furyl-acryloyl (FA)-Ala-Lys (200 μM) as substrate in 100 mM MOPS pH 7.3. Initial steady-state velocity was determined by continuous assay for a range of substrate concentrations at 340 nm with a Beckman DU 650 spectrophotometer. One unit of activity was defined as the amount of enzyme that released 1 μmol of the residue being cleaved per min at 25 °C. Kinetic parameters for recombinant *Tb*MCP-1 were also determined using FA-Ala-Lys in 100 mM MOPS buffer, pH 7.3.

The substrate preference with respect to P1′ position was determined using benzyloxycarbonyl-Ala-X (ZAX) substrates (Bachem and Sigma), using the ninhydrin method for detection [16]. The reaction mixture contained 6 mM ZAX substrate in 100 mM MOPS,