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Substrate specificity profiling of M32 metallocarboxypeptidases from *Trypanosoma cruzi* and *Trypanosoma brucei*

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ABSTRACT

Metallocarboxypeptidases (MCPs) of the M32 family, while broadly distributed among prokaryotic organisms, have so far been only found in a few eukaryotes including trypanosomatids. Among these organisms are human and animal pathogens of medical relevance such as *Trypanosoma brucei* and *Trypanosoma cruzi*, the respective causative agents of sleeping sickness and Chagas disease. The M32 MCP orthologues found in these parasites share 72% protein sequence identity. They also present a cytosolic localization, a similar pattern of expression and a marked preference for Arg/Lys residues at P1′. To further explore MCPs substrate specificity beyond the S1′ subsite, we employed four positional scanning synthetic combinatorial libraries (PS-SC) of fluorescence resonance energy transfer (FRET) peptides. Our results indicated that the *T. brucei* enzyme has a restricted selectivity for Phe in P1 position compared to *T. cruzi* MCP-1, which presented a wider range of substrate acceptance. The S2, S3 and S4 subsites, on the other hand, could accommodate a broad range of residues. On the basis of these results, we synthesized for each enzyme a series of FRET substrates which contained the most favourable residues in every position. In particular, for both MCPs acting on FRET pentapeptide substrates, catalytic efficiencies were ~100 times higher compared with previously described chromogenic substrates. In fact, the fluorogenic peptide Abz-LLKFK(Dnp)-OH (Abz = *ortho*-aminobenzoic acid; Dnp = 2, 4-dinitrophenyl) described here can be used to monitor accurately *Tb*MCP-1 activity in parasite cell-free extracts. These results provide valuable insights to develop selective substrates and inhibitors, to further understand the mechanisms and functions of M32 MCPs.

1. Introduction

Carboxypeptidases (CPs) are a group of hydrolases that perform many diverse physiological functions by removing C- terminal amino acids from proteins and peptides. These enzymes are important mediators of cellular behavior that can alter protein functionality and participate in proteome turnover [1] including vast and diverse processes such as protein trafficking, subcellular anchoring and the formation of macromolecular complexes [2,3]. CPs also modulate different signaling pathways and are connected to various pathological processes like carcinogenesis as well as neurodegenerative and cardiovascular diseases [1]. According to the chemical nature of their catalytic site, these hydrolases can be divided into three major classes, namely serine carboxypeptidases, cysteine carboxypeptidases, and metallocarboxypeptidases (MCPs), with the latter group usually employing zinc as a catalytic cofactor. Among MCPs, the M32 or *Thermus aquaticus* carboxypeptidase (*Taq*CP) family consists of a large number of enzymes with broad specificity. This family, which possesses the classical HEXXH motif observed in numerous zinc metalloproteases, is broadly distributed among prokaryotic organisms, but so far it has been found only in a few eukaryotes, namely some green algae and trypanosomatids [4,5].

The Trypanosomatidae family comprises several species that cause highly disabling and often fatal diseases such as sleeping sickness,

Abbreviations: Abz, *ortho*-aminobenzoic acid; Dnp, 2, 4-dinitrophenyl; FRET, fluorescence resonance energy transfer; MCP, metallocarboxypeptidase; MOPS, 3-(N- morpholino)propanesulfonic acid; PS-SC, positional scanning synthetic combinatorial; *Tb*MCP-1, *Trypanosoma brucei* metallocarboxypeptidase-1; *Tc*MCP-1, *Trypanosoma cruzi* metallocarboxypeptidase-1

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Chagas disease and Leishmaniasis. The current chemotherapy used in the treatment of these infections presents serious side effects, and in some cases has low effectiveness, which underscores the necessity to develop new chemotherapeutic compounds. In this scenario, proteases have become popular candidates for drug targets since they accomplish both housekeeping tasks common to many eukaryotes as well as functions highly specific to the parasite life style. For example, cruzipain, a cysteine protease belonging to the family C1, which is also an immunodominant antigen in human chronic Chagas disease [6], promotes mammalian cell invasion by *Trypanosoma cruzi* trypomastigotes and also plays a major role in the differentiation steps of the parasite's life cycle [7,8]. Although several cruzipain inhibitors efficiently kill the parasite [7,8], developing new drugs for this or other proteases has been a challenging task, due to issues such as the difficulty of achieving selectivity when targeting their active sites. In this context, the absence of M32 MCPs in metazoans constitutes an attractive trait considering the high specificity/selectivity potential of this family. In particular, *T. cruzi*, *T. brucei* and *Leishmania* spp. contain conserved M32 MCPs, which have been characterized [4,5,9–12]. Nonetheless, to date, no inhibitors or biological functions have been reported for these enzymes. On the basis of their biochemical properties and stage-specific expression, *L. major* M32 carboxypeptidase (*Lma*CP1) has been implicated in the catabolism of peptides and proteins to single amino acids required for protein synthesis [10]. Other possible functions have also emerged through the determination of the crystal structure of *T. cruzi* metallocarboxypeptidase 1 (*Tc*MCP-1) [11]. This enzyme shows a strong structural resemblance to archaeal, bacterial and mammalian metallopeptidases including angiotensin I- converting enzyme, neurolysin and thimet oligopeptidase [11]. These evidences, plus the restricted substrate preference of *Tc*MCP-1 [4], have also pointed out to a possible regulatory role in the metabolism of small peptides. In fact, it has been shown that *Tc*MCP-1 can produce des-Arg9 bradykinin by hydrolysis of bradykinin [9]. This peptide is known to promote the process of mammalian cell invasion by the *T. cruzi* trypomastigotes through B1 receptors [13]. Recently, two reports have suggested that M32 peptidases are secreted by trypanosomatids [14,15] , a fact that might make feasible the latter hypothesis.

In this work we extended the substrate specificity (P1 to P4 position) analysis of *Tc*MCP-1 and *Tb*MCP-1, two trypanosomatid M32 MCPs that remove basic C-terminal residues, using positional scanning synthetic combinatorial (PS-SC) libraries of fluorescence resonance energy transfer (FRET) peptides. These results might contribute to generate new tools to identify the roles these proteases play in the parasite biology as well as to aid in the development of selective assays and specific inhibitors.

2. Materials and methods

2.1. Enzymes

*Tc*MCP-1, *Tb*MCP-1 and *Tb*MCP-1 A414M were expressed as GST fusion proteins in *E. coli* BL21 (DE3) Codon Plus and purified as previously described [9,11].

2.2. Synthesis of peptide libraries

PS-SC libraries were synthesized by the methods previously described [16,17] except that the Abz (*ortho*-aminobenzoic acid) and Dnp (2, 4-dinitrophenyl) groups were used as the fluorescence donor and acceptor pairs, respectively. For preliminary experiments, we employed a library with the general structure Abz-GXXZXK(Dnp)-OH, where the Z position was successively replaced with one of the 19 amino acids (Cys excluded) and X represents a randomly incorporated residue introduced by coupling a balanced mixture of 19 amino acids [18]. The other three libraries presented the structures Abz-GXXRZK(Dnp)-OH, Abz-GXZRXK (Dnp)-OH, and Abz-GZXRXK(Dnp)-OH, in which two of the positions were fixed: the P2 position as Arg and Z as P1, P3, and P4 which included 19 naturally occurring amino acids with the other positions randomized (Cys excluded). For synthesis details see reference [18]. Stock solutions of each peptide mixture were prepared in DMSO, and the concentrations were measured using the absorbance of Dnp, with a molar extinction coefficient ε_{365nm} of 17 300 $M^{-1}\,cm^{-1}$.

2.3. Screening of peptide libraries

Peptide libraries were screened at 25 nM final concentration to ensure the hydrolysis of the K(Dnp)-OH group to be directly proportional to the specificity constant, k_{cat}/K_M. Reactions were initiated by the addition of the enzyme (50–100 nM) and monitored in a Hitachi F-2000 fluorescence spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan) by measuring the fluorescence at 420 nm (λ_{em}) and 320 nm (λ_{ex}). Assays were done at 25 °C in 0.1 M MOPS (3-(N-morpholino)propanesulfonic acid) buffer pH 7.3. For k_{cat}/K_M estimation, only data in the linear portion of the progression curve was considered (first order condition: $[S] << K_M$) [16].

Optimal substrates (purity > 95%) for both MCPs were synthesized by GenScript (Piscataway, NJ, USA). Assays were performed at 25 °C, in 0.1 M MOPS pH 7.3. Enzymatic activity was continuously followed in an AMINCO®-Bowman Series 2 spectrofluorometer (Thermo Spectronic, Madison, WI, USA) by measuring the fluorescence at 420 nm (λ_{em}) and 320 nm (λ_{ex}). The enzyme concentration for the initial rate determination was chosen so that less than 5% of the substrate present was hydrolyzed. The slope was converted into micromoles of substrate hydrolyzed per minute based on a calibration curve obtained from complete hydrolysis of each peptide. The kinetic parameters K_M and k_{cat} were calculated using GraphPad Prism software (GraphPad Software Inc, La Jolla, CA, USA).

2.4. Trypanosome culture

Bloodstream form *T. brucei brucei* cell line 90:13 (tetR-HYG T7RNAPOL-NEO) [19] was cultured at 37 °C under 5% CO_2 in HMI-9 medium (Sigma- Aldrich, St. Louis, MO, USA) [20] supplemented with 10% fetal bovine serum plus 2.5 μg/mL of G418 (Thermo Fisher Scientific, Waltham, MA, USA) and 5 μg/mL of hygromycin B (InvivoGen, San Diego, CA, USA).

2.5. Generation of TbMCP-1 null mutant cell lines

For a detailed description of *TbMCP-1* gene replacement cassettes, see Supplementary Material. For each transfection, approximately 2×10^7 trypanosomes grown to mid log phase ($\sim 0.8–1.0 \times 10^6$ cells/mL) in HMI-9 medium, were collected, washed once in citomix (2 mM EGTA, 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4 pH 7.6, 25 mM HEPES pH 7.6, 5 mM $MgCl_2{\cdot}6H_2O$, 0.5% glucose, 100 μg/mL BSA, 1 mM hypoxanthine) at room temperature and resuspended in 500 μL of the same buffer. About 5–15 μg of linearized plasmid was mixed into the cell suspension and transferred to a BTX 0.2 cm electroporation cuvette. Electroporation was immediately performed at a single pulse with a BTX 600 Electro Cell Manipulator (BTX Inc., San Diego, CA, USA) at 1.1 kV, 25 μF and R6 resistance (186 Ω). The entire cell-DNA mixture was transferred to a flask containing 5 mL of fresh HMI-9 medium and incubated 6 h at 37 °C without selection. Cells were diluted to a density of 2×10^5 cell/mL and 1 mL cell aliquots were distributed in 24-well plates. Selection was achieved by the addition of 2.5 μg/mL G418, 5 μg/mL hygromycin B plus 10 μg/mL of blasticidin S (InvivoGen) and/or 0.1 μg/mL of puromycin (InvivoGen).

2.6. Preparation of parasite extracts

T. brucei bloodstream trypomastigotes were broken by three cycles of freezing at −20 °C and thawing. The parasite pellets were extracted