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Analysis of peptidase activities of a cathepsin B-like (TcoCBc1) from *Trypanosoma congolense*

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ABSTRACT

The substrate specificity of TcoCBc1 was evaluated using two internally quenched fluorescent peptide libraries with randomized sequences designed to detect carboxydipeptidase (Abz-GXXZXK(Dnp)-OH) and endopeptidase (Abz-GXXZXXQ-EDDnp) activities at acidic and neutral pHs, respectively. All the data obtained with TcoCBc1 were compared with those of human cathepsin B, including the pH profiles of the hydrolytic reactions. The most relevant observation is the preference of TcoCBc1 for substrates with a pair of acidic amino acids at positions P_2 and P_1 for its carboxydipeptidase activity and the well acceptance for E and D at P_1 position for endopeptidase activity. These peculiar preferences for negatively charged groups of TcoCBc1 and its requirements for carboxydipeptidase activity were also observed on Abz labeled analogues of bradykinin (Abz-RPPG$^{\downarrow}$FSAFR-OH, Abz-RPPG$^{\downarrow}$FS$^{\downarrow}$AF-OH, Abz-RPPG$^{\downarrow}$DE$^{\downarrow}$AF-OH) and angiotensin I (Abz-DR$^{\downarrow}$VYIHAFHL-OH), where $^{\downarrow}$ indicates the cleavage site. TcoCBc1 was modeled based on the atomic coordinates of the cathepsin B from *Trypanosoma brucei* and the positively charged environment in TcoCBc1 catalytic site contrasts with the negatively charged environment in human cathepsin B. The preferences of S_1 and S_2 subsites of TcoCBc1 for acidic amino acids have to be taken into consideration for future studies of physiological roles of TcoCBc1 as for instance in apoptotic processes of *Trypanosoma congolense*.

1. Introduction

The major proteolytic activity in parasites of endemic trypanosomiasis comes from cathepsin L-like and cathepsin B-like enzymes, both pertaining to clan CA cysteine peptidase. Cathepsin L-like peptidase presents a higher activity when compared to cathepsin B-like, however, studies using RNA interference showed that the cathepsin B-like enzyme silencing in *Trypanosoma brucei* resulted in lethal phenotypes whereas the interference of cathepsin L-like expression had no effect [1,2]. Similarly, in vivo inhibition studies in *Trypanosoma congolense* using a cell-permeable cathepsin B inhibitor (CA074Me) demonstrated that cathepsin B-like enzymes are involved in lysosomal protein degradation and are essential for survival of the parasite bloodstream form [3]. *T. congolense* has an intriguing family of cathepsin B-like cysteine proteases that consists of at least 13 genes that are located on different chromosomes and exhibit significant polymorphism [3]. Similar to mammal cathepsin B, the parasite enzymes have the occluding loop, signal peptide, propeptide, the conserved Gly-Cys-Xaa-Gly-Gly motif and a catalytic domain containing the mature active enzyme. Six of these genes code for cathepsin B-like proteins with the canonical catalytic cysteine (TcoCBc 1 to 6) and in the other sequences, serine replaces this cysteine (TcoCBs7 to 12) (nomenclature is from [3]). Similar substitution was observed in several serine repeat antigens (SERAs) of *Plasmodium* parasites that are a family of secreted proteins containing a cysteine protease-like domain [4,5]. The carboxydipeptidase activities of TcoCBc1 and TcoCBc6 were demonstrated [3] with the fluorescence resonance energy transfer (FRET) peptide Abz-FRAK(Dnp)-OH [Abz = *ortho*-aminobenzoic acid, the fluorescent group and K(Dnp)-OH = (ε-NH_2-2,4-dinitrophenyl) lysine, the fluorescence quencher group] that was previously used to evaluate carboxydipeptidase activities [6,7]. The commercial fluorescent peptides Z-FR-MCA and Z-RR-MCA are efficient substrates for mammalian cathepsins [8,9], and TcoCBc1 was also able to hydrolyze them [3].

Peptidases with restricted specificity require specific peptide substrates and it seems to be the case of a cathepsin B-like protease family unique to *T. congolense*. It is important to remember that mammalian cathepsin B has carboxydipeptidase activity in the acidic compartment of lysosomes, but this activity decreases above pH 5 due to release of the enzyme occluding loop that occurs at neutral pH [10,11] and then

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the cathepsin B is able to act as an endopeptidase. In this report we present our studies on the carboxydipeptidase and endopeptidase specificities of TcoCBc1 using two internally quenched fluorescent peptide libraries with randomized sequences designed to detect carboxydipeptidase [12] and endopeptidase [13] activities at acidic and neutral pHs, respectively. The library for evaluation of carboxydipeptidase activity has the general structure Abz-GXXZXK(Dnp)-OH with free C-terminus carboxylate group and for endopeptidase activity the library structure was Abz-GXXZXXQ-EDDnp. In these libraries X consists of an equimolar mixture of all amino acids, the Z position is fixed with one of the proteinogenic amino acids and Q-EDDnp (glutamine-[N-(2,4-dinitrophenyl)-ethylenediamine] – the fluorescence quencher group). In the present work the carboxydipeptidase and endopeptidase activities of TcoCBc1 could be compared with that of human cathepsin B (hcatB) because we previously assayed the human enzyme with Abz-GXXZXK(Dnp)-OH library [12] and in the current work we assayed hcatB with Abz-GXXZXXQ-EDDnp library. In order to further investigate the carboxydipeptidase activity of TcoCBc1 we assayed it with longer peptides with sequences analogue to bradykinin (Abz-RPPGFSAFR-OH, Abz-RPPGFSAF-OH, Abz-RPPGDEAF-OH) and angiotensin I (Abz-DRVYIHAFHL-OH). In addition, TcoCBc1 was modeled based on the atomic coordinates of the cathepsin B from *T. brucei* determined at 2.55 Å resolution [14] and the general structure and the active site could be compared with those of hcatB.

2. Experimental

2.1. Enzymes

Recombinant TcoCBc1 and human recombinant cathepsin B (hcatB) were obtained as previously reported [3,10]. The molar concentrations of this enzyme were determined by active-site titration with the proteinase inhibitor trans-epoxysuccinyl-l-leucylamido-(4-guanidino) butane according to [15]. Angiotensin converting enzyme (ACE) from rabbit lung was purchased from Sigma, Saint Louis, USA (batch 040M7410).

2.2. Peptides

All the FRET peptides and Abz labeled analogues of bradykinin and angiotensin I were obtained by the solid-phase peptide synthesis strategy as previously described [16]. Briefly, the peptides were obtained by the solid-phase technique, using the fluoren-9-ylmethoxycarbonyl (Fmoc) methodology, all protected amino acids were purchased from Calbiochem-Novabiochem (San Diego, CA, USA) and the syntheses were performed in automated bench-top simultaneous multiple solid-phase peptide synthesizers (PSSM 8 system; Shimadzu, Tokyo, Japan). The final de-protected peptides were purified by semi-preparative HPLC using an Econosil C-18 column (10 μm particle size, 22.5 mm × 250 mm) and a two-solvent system: (A) trifluoroacetic acid/water (1:1000, v/v) and (B) trifluoroacetic acid/acetonitrile/water (1:900:100, by vol.). The column was eluted at a flow rate of 5 ml min^{-1} with a 10 to 50% gradient of solvent B over 45 min. Analytical HPLC was performed using a binary HPLC system (Shimadzu) with a SPD-10AV UV–vis detector (Shimadzu) and a fluorescence detector (RF-535: Shimadzu), coupled to an electron spray mass spectrometer LCMS-2010EV equipped with the ESI-probe (Shimadzu, Japan) for molecular mass determination. The concentrations of the peptide solutions were determined by colorimetric analysis of the Dnp group (molar absorption coefficient at 365 nm was 17,300 M^{-1} cm^{-1}).

2.3. Synthesis of libraries Abz-GXXZXK(Dnp)-OH, Abz-GXZRXK(Dnp)-OH, and Abz-GXXRZK(Dnp)-OH

The peptide libraries were synthesized as previously described [12], and briefly the first randomized coupling (X) was done in the free α-amino of Lys(Dnp)-NovaSyn-TGA resin using a 10-fold excess of an isokinetic mixture of Fmoc amino acids [17], with the same excess of 2-(1H-bezotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and N-hydroxybenzotriazole (HOBT) as coupling reagents. A 20-fold excess of N-methylmorpholine was added as the organic base for this reaction. The coupling of the fixed amino acid (Z) in the library Abz-GXXZXK(Dnp)-OH was done by distributing 0.1 mmol of Fmoc-XK(Dnp)-resin in 19 reaction vessels, and the synthesis was continued by coupling in each vessel 1 of the 19 Fmoc amino acids using the same excess of reagents (cysteine was excluded). Similar procedures were used for the other two libraries. The couplings of randomized isokinetic mixtures of amino acids, as well as glycine (which was a common amino acid in all mixtures), were done in each of 19 separate vessels. The Abz was added as Boc-Abz, and the mixtures of each sublibrary were removed from the resin in each vessel by treatment with TFA-1,2-ethanodithiol (EDT)-Anisol-H_2O (92.0:0.2:0.8:7.0) for 8 h. The resin was filtered and washed with 95% acetic acid, and the resulting liquid mixtures were lyophilized three times, dissolving in H_2O between each lyophilization step.

2.4. Synthesis of Abz-GXXZXXQ-EDDnp libraries

These libraries were synthesized as previously described [13], and briefly they were synthesized by solid-phase methodology starting from NovaSyn TG resin (Novabiochem, EMD Biosciences) containing the linker p-((R,S)-(1-(9-H-fluoren-9-yl)-methoxyformamido)-2,4-dimethoxybenzyl)-phenoxyacetic acid (Fmoc-2,4-dimethoxybenzyl)-phenoxyacetic acid. After removing the Fmoc group from the linker the amino acid Fmoc-Glu (γ-OH)-EDDnp was coupled by the γ-carboxyl group. Further steps were performed as described above for the library Abz-GXXZXK(Dnp)-OH.

2.5. Hydrolysis of FRET peptides

The hydrolysis of FRET peptides were quantified using a Hitachi F-2500 spectrofluorimeter by measuring the fluorescence at 420 nm following excitation at 320 nm. The inner-filter effect was corrected as previously described [18]. The concentration of DMSO in assay buffers was kept below 1%. The assays were performed in buffer containing 100 mM sodium acetate, 200 mM NaCl, 2.5 mM DTT, and 5 mM EDTA (pH 4.5 at 37 °C).

2.6. Hydrolysis of bradykinin and angiotensin I analogues

The bradykinin analogues Abz-RPPGFSAFR-OH (24 μM), Abz-RPPGFSAF-OH (30 μM) and Abz-RPPGDEAF-OH (40 μM) were incubated with TcoCBc1 (130 to 180 nM) in 100 mM sodium acetate with 2.5 mM DTT for 0 h to 20 h. After each time an aliquot was collected and the reaction stopped with 5% TFA. These aliquots were injected in an HPLC column (Shim-pack XR-ODS 4.6 mm I.D. 75 mm, Shimaduzu) and a two-solvent system: (A) trifluoroacetic acid/water (1:1000, v/v) and (B) trifluoroacetic acid/acetonitrile/water (1:900:100, by vol.) were used for a gradient from 0% to 80% of B in 40 min, with flow of 1 mL/min for developing the chromatography.

Rabbit lung angiotensin converting enzyme, Sigma-A6778 (0.3 to 0.7 nM) was incubated with each one of the bradykinin analogues in 100 mM Tris/HCl containing 50 mM NaCl and 10 μM $ZnCl_2$ at pH 8.0. The analyses by HPLC of the fragments were done in the aliquots as performed with TcoCBc1. The angiotensin I analogue Abz-DRVYIHAFHL-OH (30 μM) was incubated with TcoCBc1 and the reaction was analyzed as described with bradykinin analogues. All the peptides have the fluorescent group Abz at the N-terminus that facilitated the detection of the fragments in the HPLC system, equipped with a spectrofluorimeter, measuring the fluorescence at 420 nm following excitation at 320 nm. The identification of each fragment eluted in the HPLC was done by