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Specific negative charges in cysteine protease isoforms of *Leishmania mexicana* are highly influential on the substrate binding and hydrolysis

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Abstract

We focused on the importance of the electrostatic environment on the catalytic properties of the *Leishmania mexicana* CPB recombinant isoenzymes (rCPB2.8, rCPB3 and its mutant rH84Y), by investigating the influence of pH and NaCl on their hydrolytic activities. rCPB2.8 contains the residues Asn60, Asp61 and Asp64; rCPB3 presents the three variant residues Asp60, Asn61 and Ser64 and the mutant of the latter isoform, rH84Y, has a mutation on the outer loop residue (His84 to Tyr). Synthetic fluorescence resonance energy transfer (FRET) peptides, which contain different positive charge distribution in their sequences were used as substrates. The results show that hydrolytic efficiency is dependent of the positive charge distribution in the substrates and that NaCl activated rCPB2.8 and rCPB3 in acidic pH but inhibited them at pH higher than 5. The rate constants of substrate diffusion (k_1), substrate dissociation (k_{-1}), acylation (k_2) and deacylation (k_3) and the corresponding activation energies and entropies were derived. Significant differences in the kinetic rate constants (k) and entropies were found between the CPB isoforms, and the diffusion process seems to be the limiting step. The activation energy of denaturation ($E_{a\text{-Den}}$) and entropy of denaturation (ΔS_{Den}) of rCPB3 were higher than those for rCPB2.8, suggesting higher salvation and protein structure for rCPB3. Thus the findings suggest that the two CPB isoenzymes with a few negative charge modifications provide the parasite with an array of hydrolytic activity and enzymatic adaptation to pH, salinity and temperature that may be needed for its interaction with the mammalian host.

Keywords: *Leishmania mexicana*; Cysteine protease; Fluorogenic; FRET peptides

1. Introduction

Leishmania mexicana possesses three CPs of the papain superfamily, designated CPA and CPB, both of which are cathepsin L-like, and CPC, which is cathepsin B-like [1]. The CPB proteases exist as multiple isoenzymes, which are encoded by a tandem array of 19 similar *CPB* genes located in a single locus [2,3]. The first two genes of the array, CPB1 and CPB2, are atypical because they encode enzymes that lack the C-terminal domain, which is characteristic of this group of trypanosomatid enzymes and present in all the other CPB isoforms [3,4]. The role of their C-terminal domain remains uncertain [1,5]. *L. mexicana* Δ*cpb* mutants have reduced virulence [2,6], and only the re-expression of multiple *CPB* genes from a cosmid significantly restored virulence [7].

A recombinant form of the enzyme encoded by *CPB2.8* but lacking the C-terminal extension, originally designated

Abbreviations: Abz, *ortho*-amino-benzoyl; AMC, 7-amino-4-methylcoumarin; *t*-Boc, *tert*-butyloxycarbonyl; DMF, dimethylformamide; DTT, dithiothreitol; EDDnp, *N*-[2,4-dinitrophenyl]-ethylenediamine; EDTA, ethylenediaminetetraacetic acid; FRET, fluorescence resonance energy transfer; Fmoc, Nα-fluoren-9-ylmethyloxycarbonyl; MCA, 4-methylcoumarin-7-amide; MCA, 4-methylcoumarin-7-amide; k_1, association constant; k_{-1}, dissociation constant; k_2, acylation constant; k_3, deacylation constant at 298.15 K; Z, carbobenxy

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Table 1
Amino acid variations in the mature domains of *L. mexicana* CPBs

ENZYME	18	60	61	64	84
rCPB2.8	D	N	D	D	H
rCPB3	D	D	N	S	H
rH84Y	D	D	N	S	Y

CPB2.8ΔCTE but herein named rCPB2.8, was expressed [8], and its substrate specificity has been studied extensively [9–12] and several peptide inhibitors have also been reported for it [13–15]. The *CPB3* gene, originally designated *cDNA CPB* as it was isolated from a cDNA library [16], is another CPB gene from the central region of the array. The corresponding protein, CPB3, when expressed in Δ*cpb* mutants was devoid of the gelatinase activity in non-denaturating gel electrophoresis that was observed for CPB2.8 [3]. These two CPB isoforms differ from each other in the mature enzyme domain in only three positions; see Table 1 for comparison. Interestingly, CPB18, which also has Asp60, Asn61 and Ser64 but also Tyr84 and Asn18 instead of the His84 and Asp18 in CPB2.8, is active towards gelatin but differs from CPB2.8 in its activity towards some short peptidyl-7-amido-4-methylcoumarin substrates [3]. A detailed analysis of substrate specificity of rCPB2.8, rCPB3 and of the mutant H84Y of rCPB (hereafter named rH84Y) demonstrated that the few amino acid variations between these isoenzymes are indeed important in modifying the substrate specificities [17]. The variation of amino acid residues 60, 61 and 64 between the isoenzymes rCPB2.8 and rCPB3 mean that there are differences in the negative charge distribution of these residues, which necessarily results in significant modifications of the electrostatic potential on the surface of the isoenzymes [17].

We focused this work on studying the influence of the electrostatic environment in the catalytic properties of the isoenzymes rCPB2.8, rCPB3 and rH84Y. We have investigated the effects of pH and NaCl on the enzymes' hydrolytic capacity using the fluorescence resonance energy transfer (FRET) peptides Abz-KLRFSKQ-EDDnp, Abz-ALRFSKQ-EDDnp, Abz-KLGFSKQ-EDDnp and Abz-KLRFSAQ-EDDnp, which differ in the positive charge distribution in their sequences. In addition, based on the temperature dependence of the Michaelis–Menten parameters k_{cat}/K_m and k_{cat} for hydrolysis of Abz-KLRFSKQ-EDDnp by the three enzymes, we evaluated the individual constants (k_1, k_{-1}, k_2 and k_3) of the hydrolytic reactions using the procedure reported by Ayala and Di Cera [18] and Judice et al. [19] which was based on the assumption that the hydrolytic process occurs as in the following scheme (1):

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP + P_1 \xrightarrow{k_3} E + P_2 \quad (1)$$

where k_1 is the substrate diffusion constant into the active site, k_{-1} the substrate dissociation constant, k_2 the acylation constant and k_3 is the deacylation constant. In addition to the determination of the k_1, k_{-1}, k_2 and k_3 values, we also obtained the activation energy values E_1, E_{-1}, E_2 and E_3 relative to the rate constants of each step of the protease catalytic reactions and the respective entropies.

2. Materials and methods

2.1. Peptides

All the FRET peptides contain *N*-[2,4-dinitrophenyl]-ethylenediamine (EDDnp) attached to glutamine, a necessary result of the solid-phase peptide synthesis strategy employed which we detailed elsewhere [20]. An automated benchtop simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system from Shimadzu) was used for the solid-phase synthesis of all the peptides by the Fmoc-procedure. The final de-protected peptides were purified by semipreparative HPLC using an Econosil C-18 column (10 μm, 22.5 mm × 250 mm) and a two-solvent system: (A) trifluoroacetic acid (TFA)/H_2O (1:1000) and (B) TFA/acetonitrile (ACN)/H_2O (1:900:100). The column was eluted at a flow rate of 5 ml/min with a 10 (or 30)—50 (or 60)% gradient of solvent B over 30 or 45 min. Analytical HPLC was performed using a binary HPLC system from Shimadzu with a SPD-10AV Shimadzu UV–vis detector and a Shimadzu RF-535 fluorescence detector, coupled to an Ultrasphere C-18 column (5 μm, 4.6 mm × 150 mm) which was eluted with solvent systems A1 (H_3PO_4/H_2O, 1:1000) and B1 (ACN/H_2O/H_3PO_4, 900:100:1) at a flow rate of 1.7 ml/min and a 10–80% gradient of B1 over 15 min. The HPLC column eluates were monitored by their absorbance at 220 nm and by fluorescence emission at 420 nm following excitation at 320 nm. The molecular weight and purity of synthesized peptides were checked by MALDI-TOF mass spectrometry (TofSpec-E, Micromass) and or peptide sequencing using a protein sequencer PPSQ-23 (Shimadzu Tokyo, Japan). The concentrations of the solutions of the substrates were determined by colorimetric determination of the 2,4-dinitrophenyl group (extinction coefficient at 365 nm being 17,300 M^{-1} cm^{-1}).

2.2. Enzymes

The recombinant isoenzymes rCPB2.8, rCPB3 and rH84Y were obtained, purified and titrated as earlier described [17].

2.3. pH dependence of kinetic parameter k_{cat}/K_m

The pH dependence studies were carried out in standard buffer solution containing 25 mM acetic acid, 25 mM MES, 25 mM Tris, 25 mM glycine and 2 mM EDTA. The pH range was 3.5–10 with adjustments made using 2N NaOH and HCl. The enzymes rCPB2.8, rCPB3 and rH84Y were pre-incubated with 2.5 mM dithiothreitol for 5 min, and all the