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Substrate specificity studies of the cysteine peptidases falcipain-2 and falcipain-3 from *Plasmodium falciparum* and demonstration of their kininogenase activity

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ABSTRACT

We studied the substrate specificity requirements of recombinant cysteine peptidases from *Plasmodium falciparum*, falcipain-2 (FP-2) and falcipain-3 (FP-3), using fluorescence resonance energy transfer (FRET) peptides as substrates. Systematic modifications were introduced in the lead sequence Abz-KLRSSKQ-EDDnp (Abz = *ortho*-aminobenzoic acid; EDDnp = *N*-[2,4-dinitrophenyl]ethylenediamine) resulting in five series assayed to map $S_3 - S'_2$ subsite specificity. Despite high sequence identity and structural similarity between FP-2 and FP-3, noteworthy differences in substrate specificity were observed. The S_1 subsite of FP-2 preferentially accommodates peptides containing the positively charged residue Arg in P_1, while FP-3 has a clear preference for the hydrophobic residue Leu in this position. The S_2 subsite of FP-2 and FP-3 presents a strict specificity for hydrophobic residues, with Leu being the residue preferred by both enzymes. FP-2 did not show preference for the residues present at P_3, while FP-3 hydrolysed the peptide Abz-ALRSSRQ-EDDnp, containing Ala at P_3, with the highest catalytic efficiency of all series studied. FP-2 has high susceptibility for substrates containing hydrophobic residues in P'_1, while FP-3 accommodates well peptides containing Arg in this position. The S'_2 subsite of both enzymes demonstrated broad specificity. In addition, radioimmunoassay experiments indicated that kinins can be generated by FP-2 and FP-3 proteolysis of high molecular weight kininogen (HK). Both enzymes excised Met-Lys-bradykinin, Lys-bradykinin and bradykinin from the fluorogenic peptide Abz-MISLMKRPPGFSPFRSSRI-NH_2, which corresponds to the Met^{375} to Ile^{393} sequence of HK. The capability of FP-2 and FP-3 to release kinins suggests the involvement of these enzymes in the modulation of malaria pathophysiology.

1. Introduction

Malaria is a disease caused by *Plasmodium* parasites and remains one of the most prevalent and persistent diseases, affecting hundreds of millions of people and causing approximately 1–2 million deaths each year [1,2]. Several proteolytic enzymes have been identified in *Plasmodium* species and appear to be involved in important aspects of the parasite biology. Among them, the papain-like cysteine peptidases derived from *Plasmodium falciparum*, the most prolific human malaria parasite, have a crucial role in the physiology of malaria parasites [3,4]. These enzymes are termed falcipains (FP) and comprise four peptidases, namely falcipain-1 [5], falcipain-2 [6], falcipain-2′ [7] and falcipain-3 [3]. Compared to papain, the prototypical cysteine protease of the family that encompasses these enzymes, the falcipains display some unique motifs. These sequences, found within the N-terminal part of the prodomain, are crucial for protein targeting to the food vacuole [4]. Also in the prodomain, it was found an inhibitory C-terminal portion containing two motifs (ERFNIN and GNFD) that are conserved in the cathepsin L [8]. The C-terminal part of the prodomain is related to inhibition, and the N-terminal part of the mature domain is required for the proper refolding of the protease. These extensions are not found in other proteases of the papain family [9]. In addition, falcipains possess a unique motif that mediates haemoglobin binding [8].

Falcipain-1 (FP-1) shares 38–40% sequence identity with falcipain-2 (FP-2) and falcipain-3 (FP-3) and has been implicated in erythrocyte invasion by merozoites [10]. Gene disruption studies

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suggest that FP-1 is not essential to the erythrocytic stage of *P. falciparum* [5,11]. Gene silencing studies have alluded to a functional biological role for FP-1 [10,12]. FP-2, FP-2′ and FP-3 are food vacuole hemoglobinases, and their joint expression, in concert with aspartic peptidases, seems to be essential for efficient haemoglobin digestion [3,7,6]. Although the parasites can survive when FP-2 expression is knocked down, the silencing of FP-3 is lethal to *P. falciparum* [13], demonstrating the importance of these proteases in the parasite's life cycle. In the late stages of its intraerythrocytic development, the parasite expresses an endogenous cysteine protease inhibitor, falstatin [14].

In the present work, we performed a comparative analysis of the $S_3 - S_2'$ subsite (according to the Schechter and Berger nomenclature [15]) specificity of recombinant FP-2 and FP-3 using fluorescence resonance energy transfer (FRET) peptides derived from the lead sequence Abz-KLRSSKQ-EDDnp (Abz = *ortho*-aminobenzoic acid; EDDnp = N-[2,4-dinitrophenyl] ethylenediamine). This peptide was designed based in the results of a solid-phase substrate library for cysteine proteases [16,17] and was cleaved only at the Arg-Ser bond by FP-2 and FP-3. Five series of peptides were generated and tested: Abz-XLRSSKQ-EDDnp, Abz-KXRSSKQ-EDDnp, Abz-KLXSSKQ-EDDnp, Abz-KLRXSKQ-EDDnp and Abz-KLRSXKQ-EDDnp (X = different amino acids). Marked differences were observed in all positions analysed, suggesting a very distinct specificity between the two enzymes.

The recent description that plasmatic kininogen can be hydrolysed by *P. falciparum* intracellularly generating active peptides [18] led us to investigate whether kinins could be generated by falcipains, suggesting a real participation of these enzymes in malaria infection. Therefore, we explored the capability of recombinant FP-2 and FP-3 to release vasoactive peptides (kinins) from a synthesised fluorogenic fragment spanning the sequence from Met^{375} to Ile^{393} of human kininogen, Abz-MISLMKRPPGFSPFRSSRI-NH_2. This peptide containing the bradykinin sequence (RPPGFSPFR) extended at its C- and N-terminal sides, allowed us to evaluate the kinins that the recombinant enzymes could release. In addition, we show by radioimmunoassay that kinins can be generated by recombinant FP-2- and FP-3-mediated proteolysis of high molecular weight kininogen (HK).

2. Materials and methods

2.1. Enzymes

The enzymes falcipain-2 (FP-2) and falcipain-3 (FP-3) were expressed in *Escherichia coli* following a previously described protocol [3,19]. The recombinant enzymes were stored in 50% glycerol at −80 °C. The molar concentrations of the enzymes were determined by active site titration with E-64 according to Barrett et al. [20].

2.2. Peptides

FRET peptides containing the fluorescent group *ortho*-aminobenzoic acid (Abz) and the fluorescence acceptor N-[2,4-dinitrophenyl]ethylenediamine (EDDnp) attached to a glutamine residue were synthesised by the solid-phase synthesis method as described previously [21]. The fluorogenic kininogenase fragment Abz-MISLMKRPPGFSPFRSSRI-NH_2, which is devoid of the quenching effect, was synthesised following the same methodology. An automated bench-top simultaneous multiple solid-phase peptide synthesiser (PSSM 8 system, Shimadzu, Japan) was used to synthesise the peptides using the Fmoc-procedure. All peptides were purified by semi-preparative HPLC on an econosil C-18 column. The molecular mass and purity of synthesised peptides were checked by amino acid analysis and MALDI-TOF mass spectrometry using a TofSpec-E from Micromass (Manchester, UK). Stock solutions of the peptides were prepared in DMSO, and the concentrations were measured spectrophotometrically using the molar extinction coefficient of the EDDnp group, 17,300 M^{-1} cm^{-1} at 365 nm.

2.3. Kinetic measurements

The hydrolysis of the FRET peptides was monitored in a Hitachi F 2000 spectrofluorometer by continuously measuring the fluorescence at $\lambda_{ex} = 320 \pm 10$ nm and $\lambda_{em} = 420 \pm 20$ nm, and photomultiplier voltage set to 700 V. The assays were performed at 37 °C in 100 mM sodium acetate buffer, pH 5.5, containing 2.5 mM dithioerythritol (DTE). The 10-mm path length quartz cuvette containing the buffer was placed in the thermostated cell compartment (37 °C) of the fluorimeter until temperature equilibrium was attained. Then, FP-2 or FP-3 were added to the cuvette and left for 5 min to pre-activate the enzymes. The assays were initiated by the addition of the substrate in concentrations bellow to 1 μM, and the increase in the fluorescence with the time was continuously recorded in a final volume of 1 ml. The substrate hydrolyzed per minute was converted into micromoles based on a calibration curve obtained from the complete hydrolysis of each peptide. The experiments were carried out at low substrate concentration where the reactions followed first-order kinetics with the rate constant k_{obs} determined by the non-linear regression data analysis Grafit Version 5 program (Erithacus Software Ltd., Horley, UK). The apparent second-order rate constant k_{cat}/K_m was calculated by dividing k_{obs} by the enzyme concentration.

2.4. Hydrolysis of the synthetic kininogen-related fluorogenic peptide

The hydrolysis of the fluorogenic kininogen peptide Abz-MISLMKRPPGFSPFRSSRI-NH_2 by FP-2 and FP-3 was analysed by liquid chromatography/electrospray ionisation mass spectrometry (LC/ESI-MS) using a Shimadzu 2010 apparatus (Shimadzu Corporation, Tokyo, Japan) with an SPD-20A UV/vis detector and an RF-10AXL fluorescence detector coupled with an Ultrasphere C-18 column (5 μm, 4.6 mm × 250 mm). FP-2 (200 nM) and FP-3 (200 nM) were incubated in 0.1 M sodium acetate, pH 5.5, for 5 h at 37 °C, allowing the complete hydrolysis of the substrate (30 μM). The enzymes were pre-activated with 2.5 mM DTE for 5 min at 37 °C, before the addition of the substrate. A sample of the reaction mixture was applied in the Ultrasphere C-18 column and eluted with two solvent systems: (A) trifluoroacetic acid/water (1:1000, v/v) and (B) trifluoroacetic acid/acetonitrile/water (1:900:100, v/v) at a flow rate of 1.7 ml/min with a 10–80% gradient over 20 min after 8 min of isocratic flow. The percentage of the formation of each fragment was calculated by estimating the peak area of the generated fragment, taking the totally hydrolysed substrate to be 100%.

2.5. Radioimmunoassay determination of kininogenase activity

The ability of recombinant FP-2 (200 nM) and FP-3 (200 nM) to generate kinins from 0.52 μg of HK (Enzyme Research Co., USA) was evaluated incubating the enzymes in 0.1 M sodium acetate, pH 5.5, at 37 °C for 1 h in a final volume of 0.08 ml. The enzymes were pre-activated with 0.5 mM DTE for 5 min, at 37 °C, before the addition of the HK. The assays in the presence of 5.0 μM of E-64 were performed under the same conditions, after 30 min pre-incubation with the inhibitor. Ethanol (3:1, v/v) was added, and the mixture was centrifuged at 1000 × *g* for 15 min. The kinin content in the