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Research paper

Foot and mouth disease leader protease (Lb^{pro}): Investigation of prime side specificity allows the synthesis of a potent inhibitor

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ABSTRACT

Foot and mouth disease virus expresses its genetic information as a single polyprotein that is translated from the single-stranded RNA genome. Proteinases contained within the polyprotein then generate the mature viral proteins. The leader protease (Lb^{pro}) performs the initial cleavage by freeing itself from the growing polypeptide chain; subsequently, Lb^{pro} cleaves the two homologues of the host cell protein eukaryotic initiation factor 4G (eIF4G). We showed that Lb^{pro} possesses specific binding sites at the non prime side from S_1 down to S_7 [Santos et al. (2009) Biochemistry, 48, 7948–7958]. Here, we demonstrate that Lb^{pro} has high prime side specificity at least down to the S'_5 site. Lb^{pro} is thus not only one of the smallest papain-like cysteine peptidases but also one of the most specific. It can still however cleave between both K↓G and G↓R pairs. We further determined the two-step irreversible inhibition ($E + I \leftrightarrow EI \rightarrow E - I$) kinetic parameters of two known irreversible epoxide-based inhibitors of cysteine proteinases, E64 and CA074 on Lb^{pro} that show for the reversible step ($E + I \leftrightarrow EI$) $K_i = 3.4$ μM and 11.6 μM, and for the irreversible step ($EI \rightarrow E-I$) $k_4 = 0.16$ and 0.06 min^{-1}, respectively. Knowledge of the Lb^{pro} specificity led us to extend E64 by addition of the dipeptide R–P. This compound, termed E64-R-P-NH_2, irreversibly inhibited Lb^{pro} with a $K_i = 30$ nM and $k_4 = 0.01$ min^{-1} and can serve as the basis for design of specific inhibitors of FMDV replication.

1. Introduction

Foot and mouth disease virus (FMDV), an animal pathogen of global importance, possesses a single-stranded RNA genome [1]. Upon release into the infected cell, the genome is immediately translated into a single polyprotein. The mature viral proteins are generated by the cleavage of the polyprotein by proteinases present within it [2]. The leader protease (Lb^{pro}), the most N-terminal protein on the polyprotein, performs the first cleavage between its own C-terminus and the N-terminus of virus protein 4 (VP4) [3].

Lb^{pro} is one of the smallest papain-like cysteine proteinases known. However, despite its small size, it is also one of the most specific enzymes of its class, with only three physiological substrates at present identified. These are the viral polyprotein (VQRKLK↓GAGQS; the arrow indicates the cleavage site) as well as the two homologues of the eukaryotic initiation factor (eIF) 4G, eIF4GI (SFANLG↓RTTLS) and eIF4GII (PLLNVG↓SRRSQ) [4]. The variation present in these cleavage sequences has defied attempts to establish a consensus recognition sequence. However, recent work with FRET peptides [5] has illuminated the specificity of Lb^{pro} non prime subsites (nomenclature according to Schechter and Berger [6]), revealing a remarkable substrate binding site extending up to subsite S_7. This contrasts sharply with the non prime subsites of papain and cysteine cathepsins whose binding sites extend only up to S_3 or S_4 [7].

The exact specificities of Lb^{pro} prime sites are in contrast still ill-defined. We therefore set out to investigate these specificities, again using as the starting point the peptide Abz-

Abbreviations: FMDV, Foot and Mouth Disease Virus; Lb^{pro}, leader protease; FRET, Fluorescence Resonance Energy Transfer; MCA, methyl-coumarin amide; E64, [1-[*N*-[(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]amino]-4-guanidinobutane; CA074, [L-3-*trans*-(Propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline; DTE, dithioerythritol; Abz, *ortho*-aminobenzoic acid; EDDnp, *N*-(2,4-dinitrophenyl) ethylenediamine]; NS-134, MeO-Gly-Gly-Leu-(2S,3S)-tEps-Leu-Pro-OH.

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KVQRKLK↓GAGQSSQ-EDDnp [Abz, *ortho*-aminobenzoic acid; EDDnp, *N*-(2,4-dinitrophenyl) ethylenediamine] containing the sequence derived from the self-processing site on the viral polyprotein [5]. The analysis of Lb^{pro} cleavage of five panels of substituted peptides showed that Lb^{pro} specificity extends down to the S'_5 position. Surprisingly, proline (P) at the P'_2 position in peptide Abz-KVQRKLK↓GPGQSSQ-EDDnp resulted in one of the best substrates for Lb^{pro}. A preference for P at the P'_2 position has also been observed in certain cysteine proteases of parasites such as cruzain (of *T. cruzi*) and *Leishmania* cysteine protease B (CPB) [8–12]. Based on this information, we also explored the fitting of P to the S'_2 subsite by molecular modelling and investigated the inhibition of Lb^{pro} by the P containing epoxide inhibitor CA074 [(N-(L-3-trans-propylcarbamoyl-oxirane-2-carbonyl)-L-isoleucyl-L-proline]. The P moiety of this inhibitor has been shown to bind to the P'_2 site of cathepsin B [13]. E64 (1-[L-N-(trans-epoxysuccinyl)leucyl]amino-4-guanidinobutane) has previously been shown to inhibit Lb^{pro} activity and reduced viral yield in infected cells [14]. In order to find lead compounds for powerful and specific inhibition of Lb^{pro} and based on its prime side specificity, we extended the canonical cysteine proteinase inhibitor E64 by addition of the dipeptides G-P-NH_2, R-A-NH_2 or R-P-NH_2 to generate the compounds E64-G-P-NH_2, E64-R-A-NH_2 and E64-R-P-NH_2 and assayed them as Lb^{pro}-inhibitors.

2. Materials and methods

2.1. Enzymes

Recombinant Lb^{pro} was expressed in *E. coli* BL21 (DE3) pLysS, purified to homogeneity as previously described [15–17]. Human recombinant cathepsin B was obtained as previously described [18,19]. Human cathepsin L and papain were obtained and purified as described earlier [20]. Recombinant human cathepsin K and V were expressed in *Pichia pastoris* and purified as described in [21]. Cathepsin S was obtained as described in [22]. The concentrations of all peptidases studied in this work were determined by inhibitor active site titration method using E64 [23].

2.2. Peptides and inhibitors

FRET peptides were synthesized by the solid-phase peptide synthesis method [24]. The molecular mass and purity of synthesized peptides were checked by analytical HPLC and by MALDI-TOF using the mass spectrometer Microflex - LT (Bruker – Daltonics, Billerica, MA, USA). Stock solutions of peptides were prepared in DMSO and the concentrations were measured spectrophotometrically using the molar extinction coefficient of 17.300 $M^{-1}cm^{-1}$ at 365 nm. The epoxide inhibitors E64, CA074 [N-(L-3-trans-propylcarbamoy-loxirane-2-carbonyl)-L-isoleucyl-L-proline and CA074Me [N-(L-3-trans-propylcarbamoy-loxirane-2-carbonyl)-L-isoleucyl-L-proline methyl ester were purchased from Calbiochem-Merck (Darmstadt, Germany). The compounds E64-G-P-NH_2, E64-A-P-NH_2 and E64-R-P-NH_2 that are hybrids between E64 and CA074 epoxide inhibitors were synthesized as follows. E64-G-P-NH_2 was obtained by the reaction of G-P-NH_2 with E64 using (HATU; 2-(1H-7-Azabenzotriazol-1-yl)–1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU) as coupling reagent, with the pH maintained between 7.5 and 8.0. E64-R-P-NH_2 and E64-R-A-NH_2 were obtained by coupling E64 to the dipeptide R–P and R-A, respectively, but attached to resin using Fmoc procedures on solid-phase synthesis [24]. E64-G-P-NH_2 E64-R-A-NH_2, and E64-R-P-NH_2 were purified by HPLC and characterized by mass spectrometry.

2.3. Kinetic measurements

Hydrolysis of FRET peptides was assayed in a Hitachi F-2500 spectrofluorimeter at 37 °C. Assays with Lb^{pro} were performed in 50 mM borate/borax buffer pH 7.8 with the enzyme being pre-activated in the presence of 2.5 mM dithioerythritol (DTE) for 5 min at 37 °C before the addition of the substrates [5]. For the cathepsins, the enzymatic assays were done in 100 mM sodium acetate pH 5.5 with 2.5 mM DTE and 2 mM EDTA at 37 °C.

Fluorescence changes were monitored continuously at $\lambda_{ex} = 320$ nm and $\lambda_{em} = 420$ nm. The enzyme concentration ranges were chosen in order to hydrolyze less than 5% of the amount of added substrates. The slope of the observed fluorescence changes was converted into micromoles of substrate hydrolyzed per minute based on a calibration curve obtained from the complete hydrolysis of each peptide. The inner-filter effect was corrected using an empirical equation as previously described [25]. The kinetic parameters K_m and k_{cat} with respective standard errors were obtained through the Michaelis–Menten equation using Grafit® software. To determine the inhibition constants of non-hydrolysable FRET peptides, the fluorogenic substrate Z-LR-MCA was used. We defined FRET peptides to be non-hydrolysable when no hydrolysis was observed after incubation with 10 nM Lb^{pro} for 24 h.

All the epoxide inhibitors-Lb^{pro} complexes were demonstrated to be irreversible because the enzyme remained inhibited after extensive dialysis procedures. The parameters of irreversible inhibition of Lb^{pro} or cathepsin L by E64 and other epoxide derivatives were obtained in the same conditions as described above using Abz-KLKGAGQ-EDDnp as substrate for Lb^{pro} and Z-FR-MCA for cathepsin L. Enzyme, substrate and inhibitors were combined and product formation was monitored by the increase of fluorescence. The continuous method was employed and the inhibitions of activated cysteine peptidases were carried out in the presence of substrate and different concentrations of each epoxide inhibitor. The substrate concentrations were kept 10-fold below the K_m values. The kinetics of inactivation of the peptidases was obtained in pseudo-first-order conditions, and the reactions were continuously monitored by fluorescence measurements of hydrolysis of the substrates. The hydrolysis progress curves in pseudo-first-order conditions were treated by non-linear regression according with the equation (1) [23].

$$[P] = (v_z/k_{obs})\,[1 - \exp(-k_{obs}\,t)] + d \quad (1)$$

where P is the product concentration (in our case it is proportional to the measured fluorescence) at a given time, v_z velocity of substrate hydrolysis for zero time and k_{obs} stands for the observed first-order rate of epoxyde-induced enzyme inactivation and d is the basal fluorescence before addition of the enzyme. The inhibitory reaction was done in different concentrations of inhibitors ([I]), and all the plots of k_{obs} vs [I] were non-linear, then the irreversible inhibitions were analysed as two-step reactions as showed in Fig. 1. The K_i and k_4 parameters were obtained by non-linear regression according with the equation (2) [23].

$$k_{obs} = \frac{k_4[I]}{[I] + K_i(1 + [S]/K_m)} \quad (2)$$

where $K_i = k_{-3}/k_3$ and [S] is substrate concentration.

2.4. Determination of the substrate cleavage sites

The scissile bonds of hydrolyzed FRET peptides were identified by isolation of the fragments using analytical HPLC and the