FEBS Letters 528 (2002) 58–62 FEBS 26463

Foot-and-mouth disease virus leader proteinase: a papain-like enzyme requiring an acidic environment in the active site

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Received 19 June 2002; accepted 1 August 2002

First published online 21 August 2002

Edited by Lev Kisselev

Abstract Foot-and-mouth disease virus leader proteinase (L^{pro}), a papain-like cysteine proteinase, has six acidic amino acids between 4 Å and 11 Å of the catalytic dyad of Cys51 and His148. In contrast, in papain and related enzymes, only one acidic residue lies within this distance. We have examined by site-directed mutagenesis the importance of each of these residues for L^{pro} self-processing and cleavage of its cellular substrate, eukaryotic initiation factor 4GI. Only substitution of the electrostatic charge of aspartate 164 affected enzyme activity. Thus, in contrast to the prototype papain, L^{pro} activity requires a negative charge 4.5 Å from the catalytic dyad. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Papain-like cysteine proteinase; Active site; Foot-and-mouth disease virus; Leader proteinase; Electrostatic interactions; Initiation of translation

1. Introduction

The foot-and-mouth disease virus (FMDV) leader proteinase (L^{pro}) has only two known physiological roles. First, the L^{pro} frees itself from the growing viral polyprotein by self-processing at its own C-terminus. Subsequently, L^{pro} cleaves the host protein eukaryotic translation initiation factor 4GI (eIF4G), a part of the complex recognizing the 5′-cap structure on eukaryotic mRNAs [1–3]. Consequently, cap-dependent protein synthesis is inhibited; viral translation is unaffected, as it initiates via an internal ribosome entry segment. The cleavage sites recognized by L^{pro} are Lys-Leu-Lys*Gly-Ala-Gly in the polyprotein and Asn-Leu-Gly*Arg-Thr-Thr in eIF4GI [4,5].

Sequence and structural comparisons show that L^{pro} is a papain-like cysteine proteinase [6]. The active sites of enzymes from this family possess a catalytic dyad of cysteine and histidine (Cys51 and His148 in L^{pro}, P-Cys25 and P-His159 in papain) which appears to function as an imidazolium–thiolate ion pair [7,8]. However, as this ion pair exists at pH values at which the enzyme is not active, dissociation of additional residues must occur in papain-like enzymes [9]. Such residues responsible for the gain of catalytic competence have not yet been determined [10,11].

The active site of L^{pro} is unusual in that it is surrounded by a number of acidic residues (Fig. 1, Table 1), which can be grouped together by the distance of their carboxyl group from the Cβ atom of the active site nucleophile. The carboxyl of Asp164 is just 4.3 Å away. A second row of three negatively charged groups is situated between 6.0 Å and 8.7 Å; these include Asp163 which orients the catalytic histidine. Finally, the two residues Glu165 and Asp166 are 11.2 Å and 11.3 Å from the Cβ atom of the active site nucleophile, respectively. All except the latter two carboxyl groups of L^{pro} are closer to the active site nucleophile than P-Asp158, the closest carboxyl group to the catalytic nucleophile in papain, which is 8.8 Å away (Table 1). In addition, the acidic residues in papain suggested to participate in the gain of catalytic competence are about 13 Å away or further [9]. These differences in the position of the acidic residues have consequences for catalysis. Substitution of Asp164 with asparagine or alanine severely affect L^{pro} activity; in contrast, replacement of the equivalent P-Ser176 with alanine only reduced papain activity two-fold when measured on a peptide substrate [12,13].

Here we show that the presence and orientation of a negatively charged residue at position 164 is important for efficient catalysis by L^{pro} and investigate the importance of other acidic residues on the activity of this enzyme.

2. Materials and methods

2.1. Plasmids

pCITE Lb^{pro}VP4VP2 (encoding FMDV $O1_k$ amino acids 29–201 of the mature Lb^{pro}, all 85 amino acids of VP4 and 78 amino acids of VP2) and pET11d Lb^{pro} (FMDV amino acids 29–201) have been described [4].

2.2. Site-directed mutagenesis

DNA fragments mutagenized by standard PCR techniques were initially cloned into pBluescript KS I and their correctness confirmed by DNA sequencing. Fragments generated by appropriate restriction enzyme digestion were then used to replace the corresponding wild-type fragment in pCITE Lb^{pro}VP4VP2 or pET11d Lb^{pro} as required.

2.3. In vitro transcription and translation

Plasmids were linearized by *Sal*I; RNA was transcribed in vitro according to [3]. In vitro translation reactions (typically 50 μl) contained 70% rabbit reticulocyte lysate (RRL; Promega), 20 μCi of ^{35}S methionine (1000 Ci/mmol, Hartmann Analytic, Germany) and amino acids except methionine at 20 μM. After preincubation for 2 min at 30°C, translation was started by addition of RNA to a typical concentration of 10 ng/μl. Aliquots (10 μl) were removed at the designated time points and the reaction was stopped by immediate transfer

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Abbreviations: eIF4G, eukaryotic initiation factor 4GI; FMDV, foot-and-mouth disease virus; L^{pro}, leader proteinase; RRL, rabbit reticulocyte lysate

0014-5793/02/$22.00
PII: S0014-5793(02)03237-4

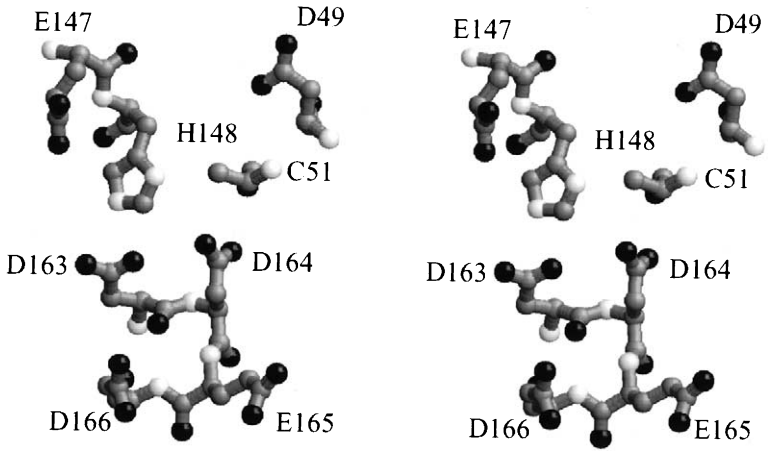

Fig. 1. Stereo view of Lb^{pro} active site. The catalytic dyad is surrounded by several acidic amino acids. The view is rotated to that in [6] so that Asp164 is at the bottom. Oxygen atoms are black, nitrogen white and carbon gray.

to ice, the addition of unlabelled methionine to a final concentration of 0.4 mM and Laemmli sample buffer (40 μl).

2.4. Polyacrylamide gel electrophoresis (PAGE) and immunoblotting

The PAGE of Dasso and Jackson [14] was used for analysis of both protein synthesis (10 μl aliquots) and the state of eIF4GI (15 μl aliquots). Translation products were detected by fluorography; the state of eIF4GI was determined by immunoblotting and detection with the anti-(eIF4GI peptide 7) antiserum as described [15].

2.5. Protein expression and k_{cat}/K_m determination

The Lb^{pro} D164E mutant protein was expressed in *Escherichia coli* BL21(DE3)LysS and purified to homogeneity as described [5]. The k_{cat}/K_m constant was determined in a fluorimetric assay using the hexapeptide VQRKLK-AMC ($\lambda_{ex} = 380$ nm, $\lambda_{em} = 460$ nm) which corresponds to the Lb^{pro} C-terminus [12,16].

3. Results

3.1. The importance of the negative charge on aspartates 163, 164 and 49

L^{pro} is the first protein encoded on the FMDV genome. As protein synthesis initiates from one of two AUG codons, two forms of L^{pro}, Lab^{pro} and Lb^{pro} can be generated [17]. Both forms have the same enzymatic activities [18]. All work described here was performed with the Lb^{pro} form.

The carboxyl groups of aspartates 163, 164 and 49 lie within 7 Å from the Cβ atom of the catalytic cysteine (see Table 1). We have previously shown that the single mutation D163N slightly reduced self-processing, without affecting eIF4GI cleavage. In contrast, the substitution D164N led to more drastic changes in both activities [12]. We wished to find out whether the charge and/or the geometry of residue Asp164 was important for enzyme activity. Furthermore, it was of interest to examine whether Asp49 was also involved in influencing the activity of the enzyme, given its similar close proximity to the active site nucleophile.

To investigate the importance of the electrostatic charge of Asp164 and its spatial orientation, we replaced this residue by glutamate (D164E). This mutation retains the negative charge; however the longer glutamate side-chain would be expected to place the charge closer to the catalytic dyad, thus possibly disturbing the geometry of the active site. RNA was synthesized from the Lb^{pro}D164E VP4VP2 mutant as well as from the wild-type plasmid and used to program protein synthesis in RRLs. Fig. 2 shows that self-processing in the wild-type is very efficient, as little unprocessed material is observed. Cleavage of the endogenous eIF4GI, which is present in the RRLs, occurs between 4 and 8 min. In contrast, self-processing in the D164E mutant was slowed, with 50% uncleaved material still present at 12 min. In addition, eIF4GI cleavage was also less efficient, with 50% cleavage occurring between 8 and 12 min (Fig. 2, Table 2). Nevertheless, the D164E mutant protein was more active than the D164N or D164A mutant proteins [12].

To confirm this result in another system, we expressed and

Table 1
Acidic residues in close proximity to the active site of FMDV Lb^{pro}

Residue	Equivalent residue in papain[a]	Mutation investigated	Distance of carboxyl from Cβ of Ala51 (Å)[b]	Distance of carboxyl from Nδ of His148 (Å)
Asp164	Ser176	D164E D164Q	4.3	4.9
Asp49	Gly23	D49N D49N/D164N	6.0	8.0
Asp163[c]	Asn175	D163N	6.8	5.1
Glu147	Asp158	E147Q	8.7	5.6
Glu165	no equivalent	E165Q E165K/D166K	11.2	11.3
Asp166	Gly185	D166N E165K/D166K	11.3	9.9

[a]Determined by superimposition of the structures of Lb^{pro} and papain [6,20].
[b]Cys51 was replaced by alanine in the crystal structure of Lb^{pro} [6].
[c]The effect of mutating residue Asp163 is described in [12].

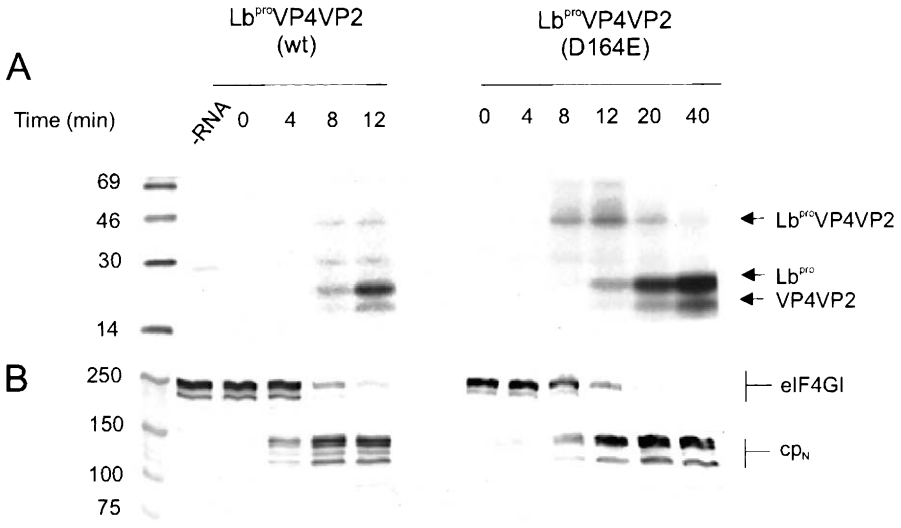

Fig. 2. Effect of the mutation D164E on Lb^{pro} self-processing and eIF4GI cleavage. RRLs were incubated with or without the indicated mRNAs (10 ng/μl) as described in Section 2; protein synthesis was terminated at the times given by placing the samples (10 μl) on ice followed by addition of unlabelled Met to 0.4 mM and Laemmli sample buffer (40 μl). Aliquots (10 μl) were analyzed for the synthesis and processing of Lb^{pro}VP4VP2 on 15% (w/v) polyacrylamide gels followed by fluorography (A); aliquots (15 μl) were analyzed on 6% polyacrylamide gels followed by immunoblotting for the status of eIF4GI (B). Fluorograms were exposed for 15 h at −70°C. The positions of uncleaved Lb^{pro} VP4VP2 and the cleavage products Lb^{pro} and VP4VP2 are marked in (A); in (B), intact eIF4GI and cleavage product (cp$_N$) are marked. Protein standards (in kDa) are shown in both panels.

purified the Lb^{pro} D164E mutant and determined its specificity constant k_{cat}/K_m on the peptide VQRKLK-AMC to be 98 M^{-1} s^{-1}, about 25-fold lower than that of the wild-type enzyme (2.65×10^3 M^{-1} s^{-1} [12]). It is worth noting that the mutant proteins Lb^{pro} D164N and Lb^{pro} D164A had no measurable activity on this peptide [12].

As the D164E mutant protein was only slightly compromised compared to the wild-type, we examined whether the introduction of the uncharged glutamine (D164Q) impaired Lb^{pro} activity to the same extent as the introduction of asparagine (Table 2; [12]). Fig. 3 shows that in the D164Q mutant protein 50% self-processing and eIF4GI cleavage are not observed until after 20 min. Clearly, the D164Q mutant protein is significantly impaired compared to the wild-type and D164E mutant proteins (compare Figs. 2 and 3). Surprisingly however, it is more active than the D164N mutant protein (Table 2).

Table 2
Effect of mutating acidic residues in close proximity to the active site of FMDV Lb^{pro}

Mutation	Time for 50% cleavage (min)	
	self-processing	eIF4GI cleavage
wild-type	<8	4–6
D164E	12–20	8–12
D164Q	20	20
D164A[a]	20	12–20
D164N[a]	20–40	40
D163N[a]	8–12	4–8
D163N/D164N[a]	>180	>180
D49N	8–10	4–8
D49N/D164N	40–60	40–60
E147Q	8	4–8
E165Q	<8	4–8
D166N	<8	4–8
E165K/D166K	<8	4–8

[a]Data taken from [12].

To investigate the influence of residue Asp49, whose carboxyl is 7.0 Å away from the Cβ atom of the active site nucleophile, we engineered the single mutant D49N and the double mutant D49N/D164N and examined the effects on enzyme activity (Table 2). The presence of asparagine at position 49 had only a minor effect on the cleavage reactions. In the D49N/D164N double mutant, self-processing and eIF4GI cleavage were severely delayed; however, this delay is not substantially longer than that seen with the D164N single mutant, suggesting that Asp49 plays only a minor role in catalysis.

3.2. *Importance of other acidic amino acids close to the active site*

Three further acidic amino acids also contribute to the unique acidic environment of the Lb^{pro} catalytic dyad. These are glutamates 147, 165 and aspartate 166 (Table 1). To investigate the role of the electrostatic charges of these residues in enzyme activity, the following mutant proteins were tested: E147Q, E165Q, D166N and the double mutant E165K/D166K (Table 2). The amide residue was chosen because it is uncharged. The double mutant E165K/D166K was designed to imitate the active site of Lb^{pro} of equine rhinitis A virus, which possesses lysine residues at these positions [19]. Of these mutants, only E147Q affected catalysis to any significant extent (Table 2). Furthermore, this effect was limited to a small reduction in self-processing, similar to that seen in the D49N mutant protein.

4. Discussion

The six acidic amino acids surrounding the catalytic dyad of FMDV Lb^{pro} give the active site of this enzyme an unusual environment (Fig. 1, Table 1). We have used site-directed mutagenesis to examine the importance of these residues for self-processing and eIF4GI cleavage. Of the six acidic amino

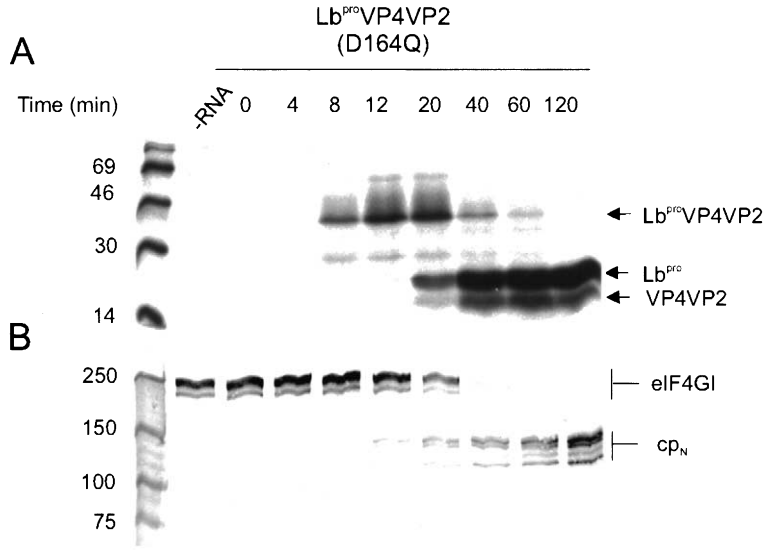

Fig. 3. Effect of the mutation D164Q on Lb^{pro} self-processing and eIF4GI cleavage. RRLs were incubated with or without mRNA (10 ng/μl). Analysis of protein synthesis (A) and the state of eIF4GI (B) was as described in Fig. 2.

acids, only mutagenesis of aspartate 164 significantly delayed the activity of the enzyme on the two natural substrates tested (Table 2). Replacement of aspartate 164 with glutamate was the most well-tolerated substitution at this position. The activity of this mutant protein is clearly higher than all other 164 substitutions tested (Table 2 [12]); furthermore, its activity, albeit low, was measurable on a hexapeptide substrate. These results imply therefore that the presence of the negative charge at residue 164 is of prime importance for the activity of the enzyme.

One surprising result was the somewhat higher activity of Lb^{pro} D164Q than Lb^{pro} D164N. As the only difference between the two residues is the additional methylene group in the glutamine side-chain, this suggests that the amide group of glutamine may extend far enough to interact with other residues. Alternatively, the longer glutamine side-chain may place the amide group out of the active site.

Of the other amino acids tested, only Asp49 affected Lb^{pro} activity, slightly reducing self-processing but without any effect on eIF4GI cleavage. The reduction was comparable to that observed when Asp163 was replaced by asparagine [12]. However, a difference between Asp49 and Asp163 became apparent when these mutations were introduced into the D164N mutant protein. The D49N/D164N mutant protein was slightly delayed compared to the single D164N mutant protein (Table 2). In contrast, the D163N/D164N mutant protein was severely compromised, with cleavage only beginning after 3 h. Thus, Asp163 appears to be more important for enzyme activity than Asp49.

In conclusion, we have determined that of the six acidic amino acids flanking the catalytic dyad, only the electrostatic charge on Asp164 and, to a lesser extent, Asp163 are important for efficient functioning of the enzyme. This represents a significant difference to the situation in papain. In this enzyme, replacement of P-Ser176, the equivalent residue to Asp164, with alanine led to only a two-fold reduction in k_{cat}/K_m when determined on a peptide substrate [13]. In addition, Lb^{pro} is the only papain-like enzyme which can be almost completely inhibited by replacement of two acidic residues, namely aspartates 163 and 164, by asparagine. This represents the best evidence yet for the presence of an electrostatic switch in a papain-like enzyme [9].

Thus, although a viral enzyme has evolved a similar fold, the mechanism of action may still differ significantly from that of cellular one. Such differences may represent a possible direction for the development of inhibitors specific for viral enzymes.

Acknowledgements: This work was supported by the Austrian Science Foundation (P-13667 to T.S.). We thank Petra Schlick and Nicole Foeger for critical reading.

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