Modulation of the electrostatic charge at the active site of foot-and-mouth-disease-virus leader proteinase, an unusual papain-like enzyme

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The leader proteinase (L^{pro}) of foot-and-mouth-disease virus is an unusual papain-like cysteine proteinase. Synthesized without an N-terminal pro precursor region, it frees itself from the growing polypeptide chain by cleavage at its own C-terminus. It also possesses a unique electrostatic environment around the active site, essentially due to Asp¹⁶³, which orients the catalytic histidine residue, and Asp¹⁶⁴; the equivalent residues in papain are Asn¹⁷⁵ and Ser¹⁷⁶. The importance of these residues for L^{pro} activity was examined by site-directed mutagenesis. Replacement of Asp¹⁶³ with asparagine reduced activity by five-fold towards a hexapeptide substrate and slightly delayed self-processing when expressed in rabbit reticulocyte lysates. However, no effect on the cleavage of the only known cellular substrate of L^{pro} , eukaryotic initiation factor 4GI (eIF4GI), was observed. In contrast, replacement of Asp¹⁶⁴ by either alanine, asparagine or lysine

abrogated activity towards the hexapeptide. Furthermore, in all cases, the onset of both self-processing and eIF4GI cleavage were significantly delayed; the reaction rates were also diminished compared with those of the wild-type enzyme. The alanine-substituted enzyme was least affected, followed by those substituted with asparagine and lysine. The double mutant protein in which both aspartate residues were replaced by asparagine was most severely affected; it failed to complete either self-processing or eIF4GI cleavage within 3 h, compared with the 8 min required by the wild-type enzyme. Hence, we propose that the electrostatic charge of Asp¹⁶⁴, and to a lesser extent that of Asp¹⁶³, is extremely important for L^{pro} to attain full activity upon synthesis.

Key words: enzyme mechanism, cysteine proteinase, ion-pair, RNA virus, translation initiation.

INTRODUCTION

Papain, a proteinase with broad specificity from the papaya plant (*Carica papaya*), is the prototype of the cysteine-proteinase superfamily [1]. Its structure and catalytic mechanism have been intensively studied [2,3], as the findings can be applied to other cysteine proteinases of medical importance. Examples of such proteinases include the caspases and cathepsins, as well as proteinases from viral and bacterial pathogens.

A viral representative of the papain-like cysteine proteinase family is the leader proteinase (L^{pro}) of foot-and-mouth-disease virus (FMDV) [4,5]. This virus possesses a single-stranded RNA genome of positive polarity; the genetic information is expressed as a single polypeptide which is subsequently processed by proteinases present within it. The L^{pro} , the first protein in the reading frame, frees itself from the polypeptide by self-cleavage between its own C-terminus and the N-terminus of the adjacent protein, VP4 [6]. As protein synthesis can initiate at one of two AUG codons lying 84 nucleotides apart, L^{pro} exists in one of two forms, termed Lab^{pro} and Lb^{pro} . Both forms possess the same enzymic activities [7].

In addition to the self-cleavage reaction, which occurs at Lys-Leu-Lys-↓-Gly-Ala-Gly, L^{pro} also processes a host-cell protein, the eukaryotic (translation) initiation factor 4GI (eIF4GI) at the sequence Asn-Leu-Gly-↓-Arg-Thr-Thr [8,9]; the effect of this cleavage is to sever the domain of eIF4GI that binds the cap-binding protein eIF4E from that which binds eIF3, which in turn binds to the 40 S ribosomal subunit. Thus the infected cell is no

longer able to recruit its own capped mRNA via the adapter molecule eIF4GI to the 40 S ribosomal subunit, effectively preventing the initiation of cellular protein synthesis [10,11]. Translation of viral mRNA is unaffected, as it initiates internally via an internal ribosomal entry site (IRES) [12].

The low overall amino acid identity (around 15%) between L^{pro} and papain indicated a distant relationship between the enzymes [4]; nevertheless, recent three-dimensional structure analysis of the Lb^{pro} form has indeed confirmed that it possesses a papain-like fold, but with an extended C-terminus [13]. This fold comprises an α -helical N-terminal domain and a β -sheet C-terminal domain, with the active-site residues positioned on opposite sides of a cleft separating the two domains [14]. The active-site residues are a nucleophilic cysteine (Cys⁵¹ in L^{pro} , Pap-Cys²⁵ in papain) and a histidine (His¹⁴⁸, Pap-His¹⁵⁹). The generation of a thiolate–imidazolium ion-pair between these two residues was thought to be sufficient for papain-like enzymes to catalyse proteolysis. Recently, however, evidence has accumulated that this ion-pair can exist at pH values at which the enzyme is not active [15–18] and that deprotonation of another site on the enzyme is required for papain-like enzymes to become catalytically competent. Several groups have investigated, by site-directed mutagenesis, residues in papain and related enzymes such as caricain (another proteinase from papaya) and ananain [from *Ananas comosus* (pineapple)] in an attempt to understand the importance of the electrostatic environment to catalysis and to pinpoint single residues which can confer catalytic competence [18–21]. To date, however, such residues have not been un-

Abbreviations used: AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulphonic acid; Caps, 3-(cyclohexylamino)propane-1-sulphonic acid; AMC, 7-amino-4-methylcoumarin; eIF, eukaryotic initiation factor; FMDV, foot-and-mouth-disease virus; IRES, internal ribosomal entry site; L^{pro} , leader proteinase; Pap-Cys (etc.), papain residue Cys (etc.); RRL, rabbit reticulocyte lysate; D163N (etc.), Asp¹⁶³→asparagine mutant (etc.).

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Figure 1 A stereo view of the network of hydrogen bonds around Asp¹⁶⁴

To ensure that all hydrogen bonds are clearly seen, the drawing is rotated compared with that in [13] along the axis of the main α -helix so that Asp¹⁶⁴ is at the bottom. Drawings were generated using MOLSCRIPT [32,33] and were rendered using raster3D [34]. Hydrogen bonds are shown by dotted lines and water molecules by small spheres.

ambiguously identified and the importance of the electrostatic environment has not been clarified [20,21].

Significant differences in the electrostatic environments of the active sites of Lb^{pro} and papain were suggested on comparison of their pH profiles [22]. When measured on the same hexapeptide substrate, papain becomes active above pH 4, whereas the Lb^{pro} remained inactive until pH 7. In contrast, the Lb^{pro} still possessed 50 % activity at pH 10, whereas papain retained 50 % activity at pH 9 and about 20 % activity at pH 10 [22]. Close scrutiny of the active sites of Lb^{pro} and papain did indeed reveal significant differences [13]. Thus Lb^{pro} has two acidic residues, Asp¹⁶³ and Asp¹⁶⁴, close to the active site; their carboxylate groups are within 6 Å and 4 Å respectively of the C β atom of the active nucleophile [22] (note: 1 Å = 0.1 nm). The equivalent residues in papain are Pap-Asn¹⁷⁵ and Pap-Ser¹⁷⁶ and are conserved in almost all papain-like enzymes [23]; therefore the environment of the thiolate-imidazolium ion-pair is much more negatively charged in the Lb^{pro} than in papain and its close relatives caracain and ananain.

This difference made the Lb^{pro} an interesting target for investigating the electrostatic environment in a papain-like enzyme. In addition, there were several other reasons justifying a mutational analysis of Asp¹⁶³ and Asp¹⁶⁴ and a determination of their influence on Lb^{pro} activity. First, Asp¹⁶³ and Pap-Asn¹⁷⁵ are the residues orienting the catalytic histidine residue in Lb^{pro} and papain; this raises the question why Lb^{pro} is the only papain-like enzyme to require an aspartate residue for this task. Secondly, both Asp¹⁶³ and Asp¹⁶⁴ are involved in networks of hydrogen bonds which are not found in papain ([13]; Figure 1). Finally, Asp¹⁶⁴ has also been proposed to play a role in binding the arginine residue of the P1' position of the eIF4GI substrate [13].

The present study investigated the effect of replacing the key residues, Asp¹⁶³ and Asp¹⁶⁴, on the activity of the Lb^{pro} form of FMDV L^{pro}; activity was monitored using the two native polypeptide substrates as well as a synthetic oligopeptide.

MATERIALS AND METHODS

Plasmids

The plasmid pET11d Lb^{pro} contains the FMDV nts 892–1411 of the FMDV serotype O_{1k} cDNA followed by two stop codons,

cloned into the *Nco*I and *Bam*HI restriction sites of the T7 polymerase expression vector pET11d (Novagen). Upon induction, the mature Lb^{pro} (FMDV amino acids 29–201) is expressed. The plasmid pCITE FMDV Lb^{pro}VP4VP2 (encoding the mature Lb^{pro}, all 85 amino acids of VP4 and 78 amino acids of VP2) was described in [24].

DNA manipulations

DNA fragments containing the desired mutations were generated using standard PCR techniques. The mutated fragments were then cleaved with the appropriate restriction enzymes and used to replace the wild-type fragments in the bacterial expression vector pET11d Lb^{pro}. The correctness of the constructions was confirmed by DNA sequencing. The mutated fragments were introduced as required by subcloning into the plasmid pCITE FMDV Lb^{pro}VP4VP2.

Protein expression and enzymic assays

Lb^{pro} wild-type and mutants were expressed in *Escherichia coli* BL21(DE3) LysS and purified to homogeneity as described in [9]. Enzymic assays using the fluorescent hexapeptide VQRKLK-7-amino-4-methylcoumarin (VQRKLK-AMC), which corresponds to the six C-terminal residues of Lb^{pro} coupled to the carboxy group of AMC, to determine the specificity constant (k_{cat}/K_m) and pH profiles were performed exactly as described in [22,25]. Briefly, measurements were performed on a Kontron SF-25 fluorimeter by monitoring the fluorescence of AMC ($\lambda_{\text{excitation}} = 380 \text{ nm}$; $\lambda_{\text{emission}} = 460 \text{ nm}$) released during hydrolysis of the substrate. Prior to the enzymic reaction, the response of the fluorimeter was calibrated by using AMC at the indicated substrate concentration. For standard assays (1 ml) used to determine the specificity constant and pH profiles, reaction mixtures consisted of 0.05 M buffer (unless otherwise stated, AMPSO {3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulphonic acid; Sigma} at pH 9.1), 1 mM cysteine, 1 mM EDTA and purified wild-type or mutant Lb^{pro} at a final concentration of 2 $\mu\text{g/ml}$. After a 2 min equilibration period,

reactions were started by addition of the hexapeptide substrate VQRKLK-AMC to 10 μ M. The dependency of activity on pH was measured using the following buffers: sodium citrate, pH 3.2 and 4.76; Mes, pH 6.0; sodium phosphate, pH 6.86; Bes, pH 7.1; Hepes, pH 7.53; Tricine, pH 8.1; Taps, pH 8.4; AMPSO, pH 9.1; glycine/NaOH, pH 9.75; 3-(cyclohexylamino)propane-1-sulphonic acid (Caps), pH 10.4, 10.8 and 11.6. As each buffer was only used at its pK value, the effect of changes in the univalent-cation concentration could be minimized. The pH of each reaction was verified immediately after measurement.

In vitro transcription and translation

Plasmids were linearized with *SalI*. *In vitro* transcription with T7 RNA polymerase and *in vitro* translation in rabbit reticulocyte lysates (RRLs) were as described in [24,26], using [35 S]methionine as the only radiolabelled amino acid. *In vitro* translation reactions (typically 50 μ l) contained 70% RRL (Promega), 20 μ Ci of [35 S]methionine (1000 Ci/mmol; obtained from the American Research Company through Humos Diagnostika G.m.b.H., Salzburg, Austria) and amino acids (except methionine) at 20 μ M. After preincubation for 2 min at 30°C, translation was started by addition of RNA to a typical concentration of 10 ng/ μ l. Aliquots (10 μ l) were removed at the designated time points and the reaction was stopped by immediate transfer to ice, the addition of unlabelled methionine and cysteine to a final concentration of 2 mM and Laemmli sample buffer.

Electrophoresis and immunoblotting

PAGE was used to separate translation products (gels contained 15% acrylamide) and to monitor the state of eIF4GI (gels contained 6% acrylamide). Translation products were detected by fluorography (exposure to film was for 15 h unless otherwise stated); the state of eIF4GI was determined by immunoblotting using the anti-(eIF4GI peptide 7) antiserum as described in [24,26].

Quantification of protein synthesis

Quantification of protein synthesis using an Instant Imager (Canberra-Packard G.m.b.H., Dreieich, Germany) was as described previously, except that calculations were adjusted for the use of [35 S]methionine alone [24,26]. Briefly, radioactivity in a particular band in the dried polyacrylamide gel was counted. By taking into account the counting efficiency (0.8%), the specific radioactivity of the methionine in the assay (163 d.p.m./fmol), the number of methionine residues in Lb^{pro} and the molecular mass of Lb^{pro} (19.8 kDa), the amount of protein present in a particular band can be estimated.

RESULTS

Mutagenesis of Asp¹⁶³ and its hydrogen-bonding partners Tyr¹⁶⁸ and Lys¹⁴⁴

To investigate the contribution of the electrostatic charge of Asp¹⁶³ on Lb^{pro} activity, site-directed mutagenesis was employed to replace Asp¹⁶³ with asparagine (D163N). Asparagine was chosen as it not only removes the electrostatic charge, but is also the residue found in almost all other papain-like enzymes except Lb^{pro} at this position. In addition, two residues which form a hydrogen-bond network with Asp¹⁶³ were also mutated [13]; Tyr¹⁶⁸ was replaced with phenylalanine (Y168F), and Lys¹⁴⁴ with glutamine (K144Q). Phenylalanine was selected to remove the hydrogen-bonding capability of Tyr¹⁶⁸ and glutamine to remove

Table 1 Specificity constants of Lb^{pro} mutants

	$10^{-3} \times \text{Specificity constant}$ [k_{cat}/K_m (M ⁻¹ s ⁻¹)]
Lb ^{pro} wild-type	2.16, 2.65*
Lb ^{pro} K144Q	2.59
Lb ^{pro} D163N	0.55
Lb ^{pro} Y168F	1.38

* Values for the wild-type are from two different preparations.

Figure 2 pH profile of Lb^{pro} mutants

Lb^{pro} activity was determined using the fluorescent hexapeptide VQRKLK-AMC as substrate, as described in the Materials and methods section. Values represent an average of three independent measurements for the wild-type (wt; ○) and the D163N mutant protein (□) and of two for the Y168F (■) and K144Q (△) mutant proteins. For comparison, the pH profile of papain measured on the same substrate [22] is shown (●). Abbreviation: rel., relative.

the charge on Lys¹⁴⁴. As the equivalent residues in other papain-like enzymes are both hydrophobic (Pap-Ile¹⁸⁷ and Pap-Leu¹³⁴), this network appears to be unique to the Lb^{pro}.

The mutations were initially introduced into the bacterial expression vector for mature Lb^{pro}, pET11d Lb^{pro}. Following induction by the addition of isopropyl β -D-thiogalactoside, the mutant Lb^{pro} molecules were purified to homogeneity as described in the Materials and methods section. The activity of the mutant proteins was then measured using the fluorescent hexapeptide VQRKLK-AMC, and the parameter k_{cat}/K_m (the specificity constant) was determined as described in [22]. The values are shown in Table 1. As it was not possible to carry out an active-site titration using *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane ('E-64'), a characteristic inhibitor of papain-like enzymes, the values must, however, be treated with caution, as they may be influenced by differences in the amount of active protein. The greatest reduction is a five-fold one with the D163N mutant protein.

Examination of the pH profiles (Figure 2) of all three mutant proteins shows that they are similar to that of the wild-type, indicating that neither the negative charge on Asp¹⁶³ nor the hydrogen-bond network around it are responsible for the differences between the pH profiles of Lb^{pro} and papain.

Previous work [22] showed that the Lb^{pro} was very sensitive to changes in the concentrations of uni- or bi-valent cations; for instance, when assayed at its optimum pH of about 9.0, the

Figure 3 Effect of mutations K144Q, D163N and Y168F on self-processing and eIF4GI cleavage activities of Lb^{pro}

RRLs were incubated with or without the indicated mRNAs (10 ng/ μ l), as described in the Materials and methods section; protein synthesis was terminated at the times given by placing the samples on ice followed by the addition of unlabelled methionine to 2 mM and Laemmli sample buffer. Aliquots were analysed for the synthesis of Lb^{pro}VP4VP2 on 15%-(w/v)-polyacrylamide gels followed by fluorography (A) and on 6% polyacrylamide gels followed by immunoblotting for the status of eIF4GI (B). All fluorograms were exposed for 15 h. The positions of uncleaved Lb^{pro}VP4VP2 and the cleavage products Lb^{pro} and VP4VP2 are marked in (A); in (B), intact eIF4GI and the cleavage product (cp_N) are marked. Protein standards (in kDa) are indicated in both panels. (C) Shows the quantification of self-processing as a histogram.

activity of Lb^{pro} was reduced by about 50% upon increasing the concentration of NaCl from 0 to 25 mM. Examination of the three mutant proteins with respect to the concentrations of uni- or bi-valent cations revealed no differences in behaviour compared with that of the wild-type enzyme (results not shown).

To confirm that the electrostatic charge on Asp¹⁶³ was not essential for Lb^{pro} activity, we decided to investigate the effect of the mutations on self-processing and the cleavage of eIF4GI, which is naturally present in the RRLs [24,26]. To this end, the mutated cDNA fragments were introduced into pCITE Lb^{pro}VP4VP2 and the corresponding RNAs transcribed. Following *in vitro* protein synthesis in RRLs, ³⁵S-labelled proteins were detected by fluorography (Figure 3A) and the state of endogenous eIF4GI in the RRL by immunoblotting (Figure 3B).

Upon translation of the pCITE Lb^{pro}VP4VP2 RNA, three labelled proteins are visible. These are the unprocessed

Lb^{pro}VP4VP2 and the products of self-processing, Lb^{pro} and VP4VP2. As self-processing is highly efficient in this system, the amount of unprocessed Lb^{pro}VP4VP2 is low. The intensity of the processed products differs because the Lb^{pro} moiety contains four methionine residues, whereas the VP4VP2 moiety has only two.

The presence of the mutations D163N and Y168F both slowed the self-processing activity of the Lb^{pro} (Figures 3A and 3C), whereas the level of self-processing with the mutant K144Q was indistinguishable from that of the wild-type. As with the assay of the purified protein mutants using the hexapeptide substrate, the presence of the D163N mutation leads to a small, but significant, reduction in activity.

The effect of the mutations on the cleavage of eIF4GI is shown in Figure 3(B). eIF4GI is a molecule which migrates on SDS/PAGE as a series of bands of molecular mass about 220 kDa. Upon cleavage by Lb^{pro}, the molecule is split into the N-terminal

Figure 4 Detailed comparison of the self-processing and eIF4G cleavage kinetics of Lb^{pro} wild-type and D163N mutant protein

RRLs were incubated with or without the indicated mRNAs (7 ng/ μ l). Analysis of protein synthesis (A) and the state of eIF4GI (B) was as described in Figure 3. Note that the fluorogram in (A) was overexposed [22] to enable the unprocessed bands to be clearly seen.

(cp_N, a series of fragments from 100 to 130 kDa) and C-terminal (cp_C, a single fragment of 100 kDa) cleavage products [27]. In these experiments, detection was performed with an antiserum raised against an oligopeptide from the N-terminal portion of eIF4GI, so that only the uncleaved protein and the N-terminal cleavage products are observed.

With the wild-type Lb^{pro}, cleavage of eIF4GI takes place between 4 and 8 min. Almost identical kinetics of eIF4GI cleavage are observed with all three mutant enzymes, even though self-processing had been impaired in two of them (Figure 3B).

To further investigate these effects, a translation experiment was performed with a lower RNA concentration over a longer time scale (Figure 4). Under these conditions, the impairment of self-processing with the D163N mutant protein is clearly visible; at the 8 min time point, uncleaved precursor represents over 50% of the synthesized material, in marked contrast with the wild-type (Figure 4A). In contrast, cleavage of the eIF4GI substrate is almost identical for wild-type and mutant (Figure 4B). The results of such an experiment with the other mutant proteins confirmed the wild-type behaviour of K144Q and a slight reduction in self-processing in Y168F (results not shown).

Importance of the residue Asp¹⁶⁴ for the activity of the L^{pro}

To investigate and define the role(s) of Asp¹⁶⁴, we carried out site-directed mutagenesis to replace it with alanine, asparagine, and lysine respectively. The double mutant D163N/D164N was also engineered to investigate the effect of removing both negative charges from the active site. The mutations were introduced into both the bacterial (pET) and RRL (pCITE) expression vectors.

The mutant proteins were first expressed in bacteria and

purified to homogeneity. During expression of the active Lb^{pro}, bacterial growth is normally slowed. However, during the expression of all four mutant proteins, the bacteria grew much more rapidly than those expressing the wild-type Lb^{pro} or those containing the single changes at position Asp¹⁶³, Tyr¹⁶⁸ or Lys¹⁴⁴ (results not shown). This suggested that the Asp¹⁶⁴ mutant proteins had a much reduced activity compared with that of the wild-type. This was confirmed by the peptide assays, as no activity could be found for any of the mutants, even though experiments were performed in which as much as ten times more protein was included in the assays than in those with the wild-type (results not shown).

The effect of the mutations on self-processing and eIF4GI cleavage was then assayed using the RRL system (Figure 5 and Table 2). The presence of each of the mutations dramatically delayed the onset and rate of both self-processing and eIF4GI cleavage when compared with the wild-type enzyme (compare Figures 4 and 5). After 8 and 12 min, self-processing of the wild-type Lb^{pro} had reached 76 and 89% respectively (Figure 3A and Table 1); eIF4GI cleavage started at 4 min and was complete by 8 min. In contrast, for each of the D164 mutant proteins, uncleaved precursor constituted the major translation product until 12 min (D164A) or even later for the other mutant proteins (Figures 5A and 5C); eIF4GI cleavage began at 8 min with the D164A and D164N mutant proteins and at 20 min with the D164K mutant protein (Figures 5B and 5D). With the double mutant, cleavage of eIF4GI did not commence until 60 min. Once initiated, however, the rates of cleavage of both substrates by the mutant enzymes did not reach those of the wild-type (Figure 5 and Table 2). Thus, even in the least affected D164A mutant protein, 50% self-processing was not achieved until 20 min after the reaction had begun. More dramatically, self-

Figure 5 Effect of mutations D164A, D164N, D164K and D163N/D164N on self-processing and eIF4GI cleavage activities of Lb^{pro}

RRLs were incubated with or without the indicated mRNAs (10 ng/ μ l). Analysis of protein synthesis (**A** and **C**) and the state of eIF4GI (**B** and **D**) was as described in Figure 3.

processing of the double mutant had not reached 50% 2 h after the reaction had been initiated. The cleavage rates of the mutant proteins on eIF4GI essentially mirrored those found in self-processing.

To confirm that the cleavage of eIF4GI was not being delayed by the inhibition of self-processing, RNAs encoding the mutant proteins were transcribed using constructions lacking the VP4/VP2 protein, generating the mature proteins upon translation.

Table 2 Effect of amino acid substitutions at D164 on Lb^{pro} self-processing and eIF4GI cleavage

Percentage self-cleavage at the time point indicated was measured as the percentage of the amount of mature Lb^{pro} present divided by the sum of the amounts of mature Lb^{pro} and uncleaved Lb^{pro}VP4VP2. The respective bands from the fluorograms in Figure 5 were counted for radioactivity in an Instant Imager and the amounts of proteins calculated using the specific radioactivity as described in the Materials and methods section.

	Time of onset (min)		Time for 50% cleavage (min)	
	Self-processing	eIF4GI cleavage	Self-processing	eIF4GI cleavage
Lb ^{pro} VP4VP2	< 8	4	< 8	4–6
Lb ^{pro} VP4VP2(D164A)	8	8	20	12–20
Lb ^{pro} VP4VP2(D164N)	12	8	20–40	20–40
Lb ^{pro} VP4VP2(D164K)	20	12–20	40–60	60–120
Lb ^{pro} VP4VP2(D163N/D164N)	60	60–120	> 180	> 180

Figure 6 Effect of the second-site mutation G202E on self-processing and eIF4GI cleavage activities of Lb^{pro} D164N

RRIs were incubated with or without the indicated mRNAs (10 ng/ μ l). Analysis of protein synthesis (A) and the state of eIF4GI (B) was as described in Figure 3.

The kinetics of eIF4GI cleavage were essentially identical with those observed when processing took place (results not shown).

Can the Asp¹⁶⁴ mutant proteins be rescued by second-site mutations?

The above mutagenesis experiments clearly indicate an important role for Asp¹⁶⁴. To investigate the role of Asp¹⁶⁴ further, we introduced additional mutations to see whether the activity of the proteinase could be rescued. The mutations were inspired by the three-dimensional structure, which indicated that Asp¹⁶⁴ may play a role in the recognition of a basic residue at the P1' position of a substrate or in the maintenance of an extensive hydrogen-bond network consisting of Asp¹⁶⁴, Asn⁴⁶, Asn⁵⁴ and Asn⁵⁰ (Figure 1; [13,22]).

Firstly, we reasoned that if Asp¹⁶⁴ were involved in recognizing a basic residue at the P1' site of the eIF4GI substrate, it might be possible to improve the activity of the D164K mutant protein by presenting an acidic residue at P1' in a substrate. As this is not feasible in the endogenous eIF4GI, we decided to perform this experiment by examining the self-processing reaction. In the wild-type, the self-processing occurs between Lys²⁰¹ and Gly²⁰², the first amino acid of VP4 in the construction Lb^{pro}VP4VP2. In previous work, we have shown that replacement of Lys²⁰¹ with

Figure 7 Effect of the second-site mutation N54D on self-processing and eIF4GI cleavage activities of Lb^{pro} D164N

RRIs were incubated with or without the indicated mRNAs (10 ng/ μ l). Analysis of protein synthesis (A) and the state of eIF4GI (B) was as described in Figure 3.

glycine reduces self-processing by about 50 % compared with the wild-type enzyme; subsequent replacement of Gly²⁰² with arginine in this mutant restored self-cleavage to wild-type levels [24]. This indicates that a basic residue can be recognized at the P1' position in self-processing as well as in eIF4GI. To examine whether residue 164 is responsible for recognizing the charge on the P1' residue, Gly²⁰² was replaced with a glutamate residue in the D164K mutant, resulting in Lb^{pro}(D164K/G202E)VP4VP2, and the activity of this mutant protein in self-processing (Figure 6A) and eIF4GI cleavage (Figure 6B) examined. However, this mutant was less able than the single mutant (compare Figures 5C and 5D with Figure 6) to carry out these reactions.

The involvement of Asp¹⁶⁴ in the hydrogen-bond network shown in Figure 1 was then investigated in a similar manner, except that the mutant D164N was employed. The presence of the amide group prevents residue 164 from simultaneously accepting hydrogen bonds from both Asn⁴⁶ and Asn⁵⁴. This interaction might be restored, however, by mutating either Asn⁴⁶ or Asn⁵⁴ to aspartate, so that the hydrogen-bond network can be re-established. The ability of the double mutant (D164N/N54D) to carry out self-processing and eIF4GI cleavage is shown in Figure 7; both activities were delayed compared with the single mutant D164N (compare Figures 5A and 5B and Figure 7). Similar results were obtained with the double mutant (D164N/N46D; results not shown). This argues against the interruption

of the hydrogen-bond network outlined in Figure 1 being the reason for the impairment of the activity of the Asp¹⁶⁴ mutant proteins.

DISCUSSION

The experiments described here were designed to investigate the roles of the residues Asp¹⁶³ and Asp¹⁶⁴ in the specific proteolysis catalysed by FMDV Lb^{pro}. Initially, the importance of the electrostatic charge of Asp¹⁶³ and the hydrogen-bond network linking it to Tyr¹⁶⁸ and Lys¹⁴⁴ were examined. Substitution of Asp¹⁶³ with asparagine led to a reduction in Lb^{pro} self-processing, but did not inhibit it completely; however, the cleavage of eIF4GI by this mutant protein was not affected. We have shown previously [24] that an Lb^{pro} protein bearing mutations at the C-terminus was, as a consequence, inhibited in respect of self-processing; nevertheless, this mutant cleaved eIF4GI at wild-type rates. The lack of a concomitant reduction in eIF4GI cleavage by the D163N mutant is thus not unusual; it does, however, suggest that self-processing places stricter constraints on the enzyme structure than does eIF4GI cleavage.

The results with the D163N mutant protein show that, in the self-processing reaction, Lb^{pro} is able to accept a residue containing either a carboxy or amide group as the residue orienting the catalytic histidine residue. In contrast, papain is inactivated in self-processing when Pap-Asn¹⁷⁵ is replaced by aspartate [28]. Possibly the inability of papain to accept the negatively charged aspartate group results from the unfavourable environment generated by the conserved tryptophan residues Pap-Trp¹⁷⁷ [13] and Pap-Trp¹⁸¹, a second conserved tryptophan residue lying perpendicular to Pap-Trp¹⁷⁷ [29]. Why is aspartate present as the third member of the catalytic triad in all L^{pro} sequences so far examined [30] when asparagine can be well accepted? Presumably even such a small reduction as that observed here would give the virus a distinct disadvantage in evolutionary terms, so that any viruses with aspartate at this position would be fitter.

No significant effect on the pH profile of the Lb^{pro} was observed on substituting Asp¹⁶³ with asparagine, Tyr¹⁶⁸ with phenylalanine or Lys¹⁴⁴ with glutamine, thus implying that these residues are not responsible for the differences between the pH profiles of papain and Lb^{pro} [22]. In addition, the effects on Lb^{pro} activity of the Y168F and K144Q mutations were modest and not detectable respectively. Taken together, these results suggest that the electrostatic charge of Asp¹⁶³ and the hydrogen-bond network around it do not play a significant role in the catalytic mechanism of the Lb^{pro}.

In contrast, the effect on the activity of the enzyme caused by removal of the electrostatic charge provided by Asp¹⁶⁴ was dramatic. Firstly, none of the Asp¹⁶⁴ mutant proteins were active on the hexapeptide substrate. Secondly, in all cases, both the rates of self-processing and cleavage of eIF4GI were considerably delayed in the D164 mutant proteins, in contrast with the results with the D163N mutant. The substitution causing the least effect was, somewhat surprisingly, alanine and not asparagine. It seems possible that the ability of an asparagine residue present at position 164 to form only one of the hydrogen bonds which can be made with aspartate may be more detrimental to the activity of the enzyme than the presence of alanine, whose methyl side chain is incapable of establishing any hydrogen bonds. An alternative explanation could be that a water molecule is present in the alanine mutant which can mimic the carboxylate more efficiently than the amide group in the asparagine mutant protein.

Given that all the Asp¹⁶⁴ mutant proteins did show some activity in self-processing and eIF4GI cleavage activity, this residue cannot be considered as acting as an electrostatic switch.

However, the loss of activity of the Asp¹⁶⁴ mutants is in contrast with the behaviour of a papain mutant in which the equivalent Pap-Ser¹⁷⁶ residue had been mutated to alanine [31]. This mutant essentially retained wild-type activity, despite the importance of Pap-Ser¹⁷⁶ in orienting Pap-Gln¹⁹, which stabilizes the oxyanion hole. Although Asp¹⁶⁴ also appears to orient the Lb^{pro} residue (Asn⁴⁶) stabilizing the oxyanion hole [13], it must be performing at least one more role than Pap-Ser¹⁷⁶.

Two experiments were performed in an attempt to determine this additional role of Asp¹⁶⁴. Firstly, we examined whether Asp¹⁶⁴ might be involved in the recognition of the P1' arginine residue of the eIF4GI substrate at Leu-Gly-↓-Arg-Thr. As it is not possible to modify the endogenous eIF4GI in the RRLs, this notion was examined using the self-processing reaction. Glycine at the P1' position of the cleavage site between Lb^{pro} and VP4 was replaced with a negatively charged glutamate residue. If Asp¹⁶⁴ can recognize a basic P1' residue, it might be expected that the D164K mutant might recognize an acidic residue at this position. However, self-processing was in fact delayed in this double-mutant protein compared with the single-mutant protein, casting doubt on the role of Asp¹⁶⁴ in binding eIF4GI and reopening the question as to how Lb^{pro} in fact recognizes a basic residue at P1'.

The second experiment examined the involvement of Asp¹⁶⁴ in an extensive hydrogen-bond network which was interrupted by all of the three amino acids with which Asp¹⁶⁴ was replaced. To investigate the importance of this network, we attempted to reconstitute it in the D164N mutant by replacing either Asn⁵⁴ or Asn⁴⁶ with aspartate. Neither of the double mutants showed any increase in activity compared with the single D164N mutant, indicating that the hydrogen-bond network is not essential or that it can not be reassembled in this way.

As the above ideas were not supported by experimental evidence, another hypothesis is required to explain why the replacement of Asp¹⁶⁴ affects both onset and rate of the catalytic activity (Figure 5 and Table 2). It should be remembered that Lb^{pro}, in contrast with papain, is synthesized without a pro region. It is thus clear that Lb^{pro} has developed an extremely efficient mechanism for attaining an active conformation as it commences to cleave its two substrates immediately after synthesis. Indeed, cleavage of eIF4GI just 15 min after infection of BHK cells *in vitro* has been reported [9]. Replacement of Asp¹⁶⁴ obviously impairs this mechanism, as all Asp¹⁶⁴ mutant proteins were initially severely retarded in their enzymic activity. This suggests an important role for this residue in folding and stability. Furthermore, it is noteworthy that the two mutants which require the longest time to gain a detectable amount of activity are the D164K single mutant and the D163N/D164N double mutant. This would indicate that the repulsion of the negative charges of the two aspartate residues is indeed critical. Indeed, Asp¹⁶⁴ is itself involved in a tight type II' β -turn of unfavourable geometry; possibly it is the repulsion between Asp¹⁶⁴, Asp¹⁶³ and other as-yet-unidentified residues which is required to maintain Asp¹⁶⁴ in this state.

The above interpretation implies that the role of Asp¹⁶⁴ is to ensure correct folding, and that its role in catalysis of proteolysis is less important. That the presence of the methyl side chain of alanine at residue 164 least affected catalysis also supports the interpretation that Asp¹⁶⁴ is not involved in catalysis. Further evidence can be derived from the above-mentioned differences in kinetics of cleavage of eIF4GI by the respective D163N and D164N mutant proteins. Thus, although the D163N mutant protein is delayed in self-processing, cleavage of eIF4GI is not affected. In contrast, in the D164N mutant both cleavages are delayed [compare the 12 min time points of Figures 4A and 4B

(D163N) with Figures 5A and 5B (D164N)]. This would suggest that the D164N mutant protein is initially in an inactive form and requires a certain period to become active. This implies, therefore, that Asp¹⁶⁴ plays an important role in gain of catalytic competence, whereas the task of Asp¹⁶³ is more limited to catalysis. Structural studies on selected Asp¹⁶⁴ mutant proteins are in progress in an attempt to answer this question.

In summary, the experiments shown here reveal that the Lb^{pro} has evolved a different mechanism for stabilizing and orienting its active site from that found in the majority of the papain-like proteinases, and suggest a key role for the electrostatic charge on Asp¹⁶⁴. Once again, however, it is clear that the mechanism as to how cysteine proteinases achieve full catalytic competence still requires further investigation to be understood completely.

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