The enhancing of a cysteine proteinase activity at acidic pH by protein engineering, the role of glutamic 50 in the enzyme mechanism of caricain

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Abstract ***Carica papaya*** **produces four cysteine proteinases. Calculations show that the Cys[25], His[159] essential ion pair is fully ionised at pH 2.99, where activity cannot be detected, but apparently an additional ionisation with a pK_a of 4 is essential for activity (an electrostatic switch). Caricain (EC 3.4.22.30) wt and D158E genetic backgrounds were used to study the contribution of E50A to activity. E50 or E135 are candidates for the switch, E50A would be expected to reduce activity. However, activity increased at pH 5.0 in both backgrounds and at the pH optimum in D158E E50A but decreased slightly in the wt background. This challenges the hypothesis of an electrostatic switch.**

Key words: Kinetically influential mutant; Cysteine proteinase; Site-directed mutagenesis

1. Introduction

Cysteine proteinases are endopeptidases which have catalytic activity because of the nucleophilicity of the active site cysteine. An ion pair is formed between the active site cysteine and a histidine on the opposite side of the active site cleft.

Many of these enzymes have been extensively structurally characterised and the spatial arrangement of residues in caricain has shown that, in common with all of the plant cysteine proteinases, an arrangement of charged amino acids produces an unusually low pK_a for cysteine 25 [1–3]. The histidine, at position 159, acts as the acid/base in the reaction and is close enough to C25 to affect its ionisation by 4–5 pH units. However, the precise pK_a of ionisation of C25 has been rather obscured by data concerning the ionisation of other charged amino acids in close vicinity to position 25. It was originally argued that either an aspartic acid residue at position 158 or an asparagine at position 175 had a pivotal role in the mechanism of these enzymes, similar to the 'charge relay system' of serine proteinases. [4]. However, analysis of the electrostatic environment of C25 in plant cysteine proteinases clearly showed that other aspartic and glutamic residues as well as main-chain partial charges (in particular the helix residues 25–42) could contribute to stabilising the thiolate ion at position 25 with a low pK_a [5]. It was also obvious that, in all the plant proteinases, the k_{cat}/K_m profile vs pH could be seen to show a number of ionisations occurring between 2 and 7. This was especially clear in caricain [6,7]. A sound mathematical basis for analysing these ionisations, known as multihydronic states [8], was established by Brocklehurst [9] and has subsequently been used to analyse the kinetic affects of mutations around the active site [3,8,10–15].

Experiments using probes which react with a thiolate ion at different rates at different pH values originally were interpreted to show that ionisation occurred at a pH value of 3.4 [8] in caricain and this appeared to be borne out by steady-state kinetics [6] but with at least two other ionisation events between 4 and 7 being necessary before maximum activity was obtained. Mutagenesis experiments with papain and caricain allowed activity to be measured with glutamic acid as well as asparagine and alanine at position 158 [11,12]. The results showed that 10% of activity was still present when alanine replaced aspartic acid at position 158; this was taken to mean that aspartic acid at position 158 was not essential to the mechanism. As cathepsins B and H are cysteine proteinases without aspartics at 158 and we have recently cloned and expressed the first cysteine proteinase from plants without aspartic at 158 [13], it is clear that it must be the overall electrostatic field that produces maximum stability for the thiolate ion and it is important to define the other residues which actually contribute to increasing activity.

By probing the reactivity of cysteine 25 in the caricain mutant D158E using dipyridyl disulphides at various pH values we have shown that the cysteine ionises at low pH values (2.98) in both wild type and the D158E mutant [3]. This is despite steady-state kinetic analysis indicating that the first ionisation seen in the mutant activity profile occurs at pH 4.9 as opposed to 3.4 in the wild type [6,12]. Structural studies showed that the crystal structure of the mutant was essentially the same as that of the wild type [2,3].

We therefore postulate that the aspartic residue 158 in wild-type caricain actually has a pK_a below 3.0 and the cysteine (despite ionising at pH 2.9) has little catalytic competence until the deprotonation of the carboxyl of 158 occurs [3,14,15]. In the D158E mutant it is to be expected that the carboxyl of the glutamatic acid would ionise at a higher pK_a than the carboxyl of the aspartic acid at the same position and so activity appears at a higher pH, although the cysteine has been shown to ionise at the same pH as in the wild type. The activity that is seen in the steady-state kinetics, in both wild type and D158E, would therefore depend on (1) the ionisation of cysteine and a charged residue at 158 giving a basic activity and (2) subsequent ionisation or ionisations at pH values between 4 and 7 giving a further enhancement of activity above pH 4, as seen in Figure 5 of [6] and Figure 1 of [12].

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The obvious candidates for this secondary enhancement are glutamic acids 50 and 35 [3,5]. Pinitglang et al. [15] have used data from reactivity probes and steady-state data with neutral substrates to suggest that activity in caricain may be critically dependent on the ionisation of E50. This mechanism was described as an electrostatic switch, and it was claimed that the ion pair is catalytically incompetent without the contribution of the electrostatic field from E50 (interaction energy of −52.7 kcal/mol [15]). We have therefore mutated E50 to alanine in both a wild-type background and a D158E background to test this hypothesis. It was found that the removal of the charge at position 50 produced an enhanced activity using a neutral substrate at pH 5.0 in both backgrounds. In the wild-type background the activity at optimal pH values was reduced by a small amount (when compared to wild type) but increased in the double mutant (when compared to D158E). At pH 6.5 the k_{cat} was reduced in both mutants (when compared to both wild type and D158E) but K_m was unaltered in the single mutant. However, in the double mutant the K_m was markedly improved (compared again to both the wild type and D158E). Thus removal of charge at position 50 has identified a restraint on activity at low pH using a neutral substrate. In the light of results published earlier [6] it would appear that ionisation of E50 at approximately pH 4.8 has an affect upon K_m. This confirms that glutamic acid at position 50 interacts strongly with the active site as predicted but seems to disprove the theory that the ion pair is catalytically incompetent when E50 is an uncharged residue.

2. Materials and methods

2.1. Site-directed mutagenesis by inverse PCR

Caricain clone X66060 was the starting material for mutagenesis. As explained [12,3], the sequence used did not include the nucleotides for the first 26 amino acid signal sequence found in the native gene.

The primers used to make site-directed mutants of caricain at position 50 in both wild-type and D158E background are shown below. The characters in bold indicate the position of the mutation and we also introduced a silent mutation which produced a new *Sal*I restriction site into the mutant. Oligonucleotides were purchased from a commercial source. They carried the minimum number of base mismatches with the template to introduce the desired mutations. The template DNA, either caricain wild type or caricain D158E, was cloned in pBS (Stratagene) between an *Nde*I and a *Bam*HI site [12,16].

The oligonucleotides to introduce E50A and a new *Sal*I site were: forward: 5′-TCA **GCA** CAA GAA CTT GTC GAC TGT G-3′; reverse: 5′-TAA TTC TAC TAA TTT TCC AGT TC-3′.

In the PCR reaction we used 25 cycles of 1 min of denaturation at 94–96°C, 1 min of annealing at 50–60°C and 6–8 min of extension at 72°C. Conditions were optimised in each case, as was the magnesium concentration, and the mixture contained 2.5 units of AmpliTaq polymerase (*Thermus aquaticus*) [17] and a similar volume of proofreading polymerase from *Pyrococcus furiosus*. Mixtures were a final volume of 50 μl with 1 μg of primer. Products were tested for size on an agarose gel and samples containing a 4 kb molecule were treated for 20 min with 4.6 units of Klenow fragment and 1 μl of 25 mM dNTPs followed by 10 min at 65°C. Product was precipitated with 50 μl of 5 M ammonium acetate and 100 μl isopropanol, centrifuged and washed with 70% ethanol. Dried pellet was treated with 10 units of T4 polynucleotide kinase and 3 μl of 10 mM ATP in the buffer described in [10] at 37°C for 30 min. After thermally inactivating the kinase at 65°C the DNA was again precipitated, washed, dried and incubated with 1 unit of T4 DNA ligase for 24 h (buffer described in [12]). A DNA sample was taken and prepared for both restriction analysis and, if the new restriction site was present, sequencing. DNA was then transformed into SURE or TG1 *Escherichia coli* cells (Stratagene). 5–20 μl of the ligated product was added to 100 μl of host cell suspension and kept on ice for 40 min.

Cells were heat-shocked at 42°C for 2 min. 500 μl of LB broth was added to the cell suspension and the transformed cells grown for 45 min at 37°C. Single colonies were then grown on in the presence of antibiotic and restriction digests on the extracted DNA showed which colonies had the new restriction site. Maxipreps were used to prepare DNA which was restricted by *Nde*I and *Nco*I and sequenced. This 500 bp fragment was purified by agarose gel electrophoresis and ligated back into the original caricain clone.

The expression system was the T7 polymerase system of [18], and expression was as described by [3,12], using pET3a in BL21(DE3) cells.

2.2. Refolding and purification of recombinant procaricain

In order to produce recombinant caricain *E. coli* was grown with the pLysS plasmid according to the procedure described [3] except that overnight cultures were inoculated into 3 l of LB broth (6×500 ml flasks). After induction with 0.4 mM IPTG, cells were collected by centrifugation at 5860×*g* for 10 min at 4°C and stored at −20°C (no longer than 1 month). The cell pellet was lysed by passing through a French press and then insoluble inclusion body material was collected by centrifugation at 5860×*g* for 10 min at 4°C. The inclusion body material was solubilised and refolded using the methods and apparatus described [3]. Refolded material was concentrated about 50-fold using a Millipore Minitan filtering system with a PK 5000 NMWL high flux biomax polysulphone membrane. Final 5-fold concentration was achieved with Aquacide II (Calbiochem). The protein solution was dialysed against 50 mM sodium acetate (pH 5.0) containing 0.5 M NaCl and then centrifuged to remove the insoluble material. The solution was slowly diluted 2-fold with 50 mM sodium acetate (pH 5.0) and subsequently applied to a MonoS HR5/5 column. Elution was achieved with a gradient of 0.25–1 M NaCl in 50 mM sodium acetate buffer (pH 5.0). The peak fractions were immediately converted to active caricain by heating at 60°C for 30 min in the presence of 10 mM cysteine and 1 mM EDTA [15]. SDS-PAGE was as described by Laemmli [19] and the stacking gel was 5%, the separation gel was 12% [3,12].

2.3. Enzyme assay and kinetic analysis

Activity was determined by following the initial velocity of the release of *p*-nitroaniline at 410 nm ($E_{410} = 8.8\times10^3$ M^{-1} cm^{-1}) from the neutral substrate pyr-Glu-Phe-Leu-pNa (pEFLNA) at 25°C and over a pH range of 3.0–9.5. Buffers were as described [12] and their osmotic strengths were adjusted to 0.16 with NaCl. Final concentrations of buffer were always 50 mM and reaction mixtures also contained the following final concentrations: 1 mM EDTA, 10 mM cysteine, 0.05 mM substrate in 0.5% DMSO. Because of the insoluble nature of the substrate the concentration was kept lower than the K_m values at different pH values. The value $(k_{cat}/K_m)_{obs}$ is obtained from the relationship $v_i = e_o s\,(k_{cat}/k_m)_{obs}$ where e_o is the enzyme concentration [11,12]. In order to measure the exact concentration of caricain active sites in each reaction it was necessary to use titration. The titrant was the active site-directed irreversible inhibitor E-64 and this stoichiometrically reduced activity so that each time the assay was done we obtained the exact figure for the concentration of caricain [3]. Data were analysed using the reaction scheme shown in [12]. As extensive analysis of the kinetics of cysteine proteinases has been established with both wild-type and mutant protein, we fitted the data to the three- and four-hydronation state models shown in [12] using a non-linear data fitting program (Kaleidagraph, Abelbeck Software). This allows direct comparison with previous research. For detailed discussion of these models for kinetic analysis and the proof that they are optimal under these conditions see [8,9].

3. Results

3.1. Overexpression and purification

The DNA from both mutants were sequenced extensively, both as restriction fragments and as the complete construct. The mutations were confirmed as being present; the data are not shown but are available for inspection. The cells were grown as described and, after induction by IPTG, the enzyme was produced as inclusion bodies. During several different expression experiments the yield was about 8 g wet weight of cells/3 l broth and about 1 g of inclusion body material

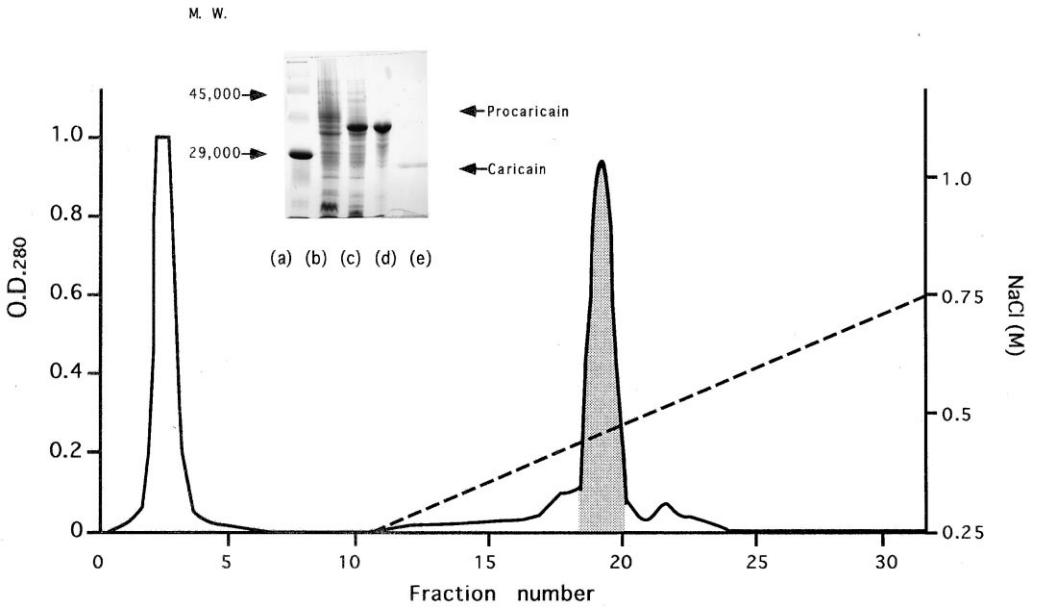

Fig. 1. Separation of proteins at various stages of heterologous expression. The separation system was FPLC using a MonoS HR5/5 column and elution was achieved using a gradient of 0.25–0.75 M BaCl in 50 mM sodium acetate. Also shown is a electrophoretic gel; lane a = molecular weight markers, lane b = proteins extracted from *E. coli* before induction with IPTG, lane c = proteins extracted from *E. coli* after induction with IPTG, lane d = proteins after inclusion body separation and redissolution in GuHCl, lane e = purified proteins from fractions 18–20 as shown in the main part of the figure.

was produced after cell lysis. The material could be purified to homogeneity by FPLC (Fig. 1).

Denaturing PAGE indicated that the active enzyme was found as two bands at the correct molecular weight (Fig. 1). This is consistent with the results of [3] who found that the larger band was an active enzyme with four or five amino acids of the pro-region attached at the N-terminus. These did not affect activity.

3.2. Data analysis

With the neutral substrate pEFLNA it was found that both mutants were active across the pH range tested.

Experimental data points at each pH were triplicated and the experiments repeated. A representative data set is shown and activity was fitted to both three and four pK_a equations described as equations 6 and 7 of [12].

The data for the single mutant were analysed using methods optimised for the wild type and which revealed most information about the hydronations involved in the mechanism [8,12]. This involved the use of four hydronations and three active species in the calculation and the single mutant E50A showed a clear change in the pH profile when compared to the wild type (Fig. 2 compared to Fig. 3).

Fig. 3 also shows the activity of D158A for comparison. The removal of charge at position 158 clearly affects activity

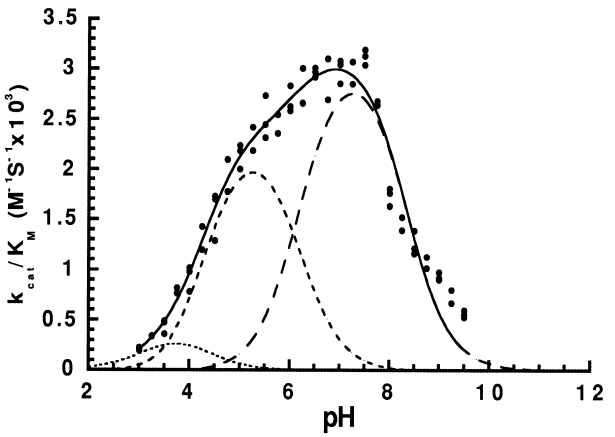

Fig. 2. A single amino acid mutant, E50A, of caricain showing the efficiency of activity (k_{cat}/K_m) vs pH. This is fitted to the equation describing four hydronations and three active species (shown in equation 6 of [12]).

Table 1
pK_a and k_{cat}/K_m^{lim} (M^{-1} s^{-1}) values for each ionization, obtained by using equations 6 and 7 of [10] for fitting the pH activity curves of the single mutant (E50A) and the double mutant (E50A, D158E) of caricain

D158E, E50A double mutant			E50A single mutant		
	pK_a or k	± S.D.		pK_a or k	± S.D.
3-Hydronic state model					
pK_a1	4.75	0.05	pK_a1	4.14	0.05
pK_a2	6.74	0.35	pK_a2	6.07	0.23
pK_a3	8.21	0.04	pK_a3	8.34	0.03
k1	1912	65	k1	2318	104
k2	2426	63	k2	3209	58
χ =	1.5889		χ =	3.2093	
reg =	0.9774		reg =	0.9739	
4-Hydronic state model					
pK_a1	4.86	0.09	pK_a1	3.15	0.27
pK_a2	5.73	0.14	pK_a2	4.32	0.43
pK_a3	6.21	0.43	pK_a3	6.19	0.33
pK_a4	8.22	0.03	pK_a4	8.33	0.03
k1	2150	331	k1	398	809
k2	1435	825	k2	2420	139
k3	2420	57	k3	3221	64
χ =	1.553		χ =	3.1744	
reg =	0.9779		reg =	0.9741	

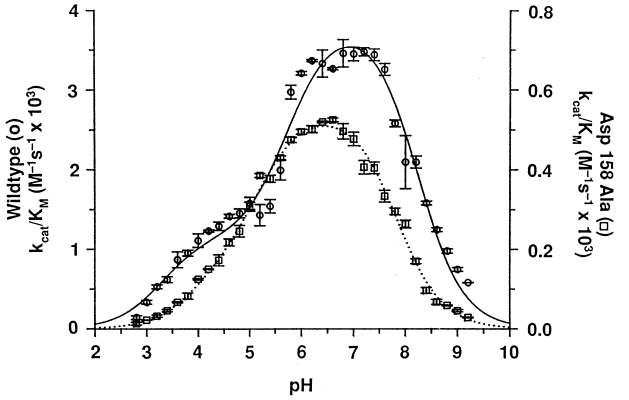

Fig. 3. Data for wild-type caricain and the mutant E158A showing the efficiency of activity (k_{cat}/K_m) vs pH, comparison with E50A is described in the text. Wild-type data are fitted to the equation describing four hydronations and three active species (equation 6 of [12]). Reproduced with permission.

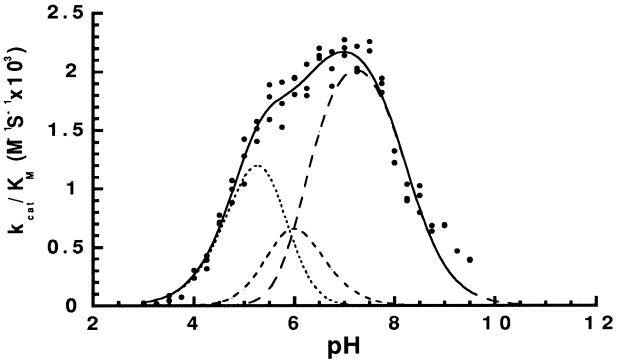

Fig. 4. A double amino acid mutant, D158E, E50A, of caricain showing the efficiency of activity (k_{cat}/K_m) vs pH. This is fitted to the equation describing four hydronations and three active species (equation 7 of [12]).

much more than removal of charge at position 50. The acid side of the pH profile of mutant E50A shows that the efficiency (k_{cat}/K_m) is considerably greater at pH values of 4–6 than in the wild type. However, the overall activity at pH 6–8 is reduced slightly from 3.5–3.0 at pH 7.0. Comparable figures for k_{cat}/K_m at pH 5.0 are ca. 2.25 for the single mutant and 1.25 for the wild type. At pH 5.5 the mutant has values of 2.5 versus 1.5 for the wild type. Thus the efficiency of activity is enhanced at acid pH in E50A.

The double mutant D158E, E50A shows a combination of the activity reported in [12] for D158E and the activity of the single mutant E50A. This interesting result indicates that the two charged residues separately affect activity in the acid portion of the profile.

At pH values of 3–4 the double mutant displays the low activity that was previously reported for D158E [12] when compared to wild type. However, in the region between pH values of 4 and 6 the double mutant displays considerably more activity than D158E. At pH 5.5 the double mutant has k_{cat}/K_m values of ca. 1.8 whereas D158E has values of ca. 0.6. The double mutant enzyme efficiency is even greater than the wild type over this pH range. Activity of the double mutant at the pH optimum is ca. 2.3 whereas D158E was 1.5 and 3.5 for wild type.

When the data are modelled using either an equation with four hydronations and three active species or three hydronations and two active species (equations 6 and 7 of [12]) the regression coefficients for both mutants are very similar. Table 1 shows that data using E50A and both models only show variation between the models at the third decimal place of the regression analysis and, in the case of D158E, E50A, the variation is only at the fourth decimal place. The four pK_a model is the preferred mathematical solution in both cases but the fit is good with both three and four pK_a fits (Table 1). Table 1 shows that the pK_a on the alkaline limb of both mutants does not vary significantly compared to the data of [3,12] or the wild type. It falls within the range of 8.2–8.22 for the D158E, E50A (compared to 8.17 for D158E) and 8.33–8.34 for the single mutant.

Table 1 shows that in the best fit case pK_a1 for E50A is 3.149 whereas the three hydronic state model has pK_a1 as 4.138. The former figure is close to that for the wild type in [12] and also to the recalculated figures of [14,15] which assign the first pK_a to the cysteine with a pK of 2.9. Thus we have confidence that the first pK_a reflects the ionisation of C25. However, the other pK_a values in the acid part of the curve are very different from those calculated for D158E or wild type. The removal of charge has produced, with this neutral substrate, a pattern of calculated pK_a values which are more widely spread than in the wild-type enzyme. Although one charged residue has been removed entirely, the closeness of the C25 pK_a to that calculated for wild type and the mutant D158E [3,12,14,15] gives confidence that the four hydronic acid species is correct.

In the double mutant the four hydronic model is the most accurate model but both solutions agree that the first pK_a is at pH 4.7–4.9 which is similar to D158E whose first pK_a is recorded as 4.93 [12] However, the other pK_a values in the acid part of the curve are different from either wild type or D158E

The four-hydronation model for the single mutant is plotted in Fig. 2 and for the double mutant in Fig. 4.

Table 2 shows that at the pH optimum, pH 6.75, the k_{cat} is reduced for both mutants below that found in the wild type; however, the value for the single mutant is not significantly different when compared to wild type or D158E. The K_m is not significantly changed in the single mutant when compared to the wild type but it is substantially better than D158E. However, the K_m of the double mutant is significantly improved when compared to either the wild type or D158E.

Table 2
Kinetic constants shown by caricain mutants D50A and D50A, D158E using the substrate pyr-EFLNA at pH 6.75 and $I = 0.16$, compared with D158E and wild-type data (from [12])

Mutant	k_{cat}	K_m	k_{cat}/K_m (M^{-1} $s^{-1} \times 10^3$)
Wild type	4.41 ± 0.061	1.18 ± 0.19	3.70 ± 0.79
E50A	3.90 ± 0.20	1.28 ± 0.20	3.05 ± 0.06
D158E, E50A	1.57 ± 0.13	0.75 ± 0.05	2.08 ± 0.08
D158E	3.65 ± 0.78	2.61 ± 0.18	1.4 ± 0.04

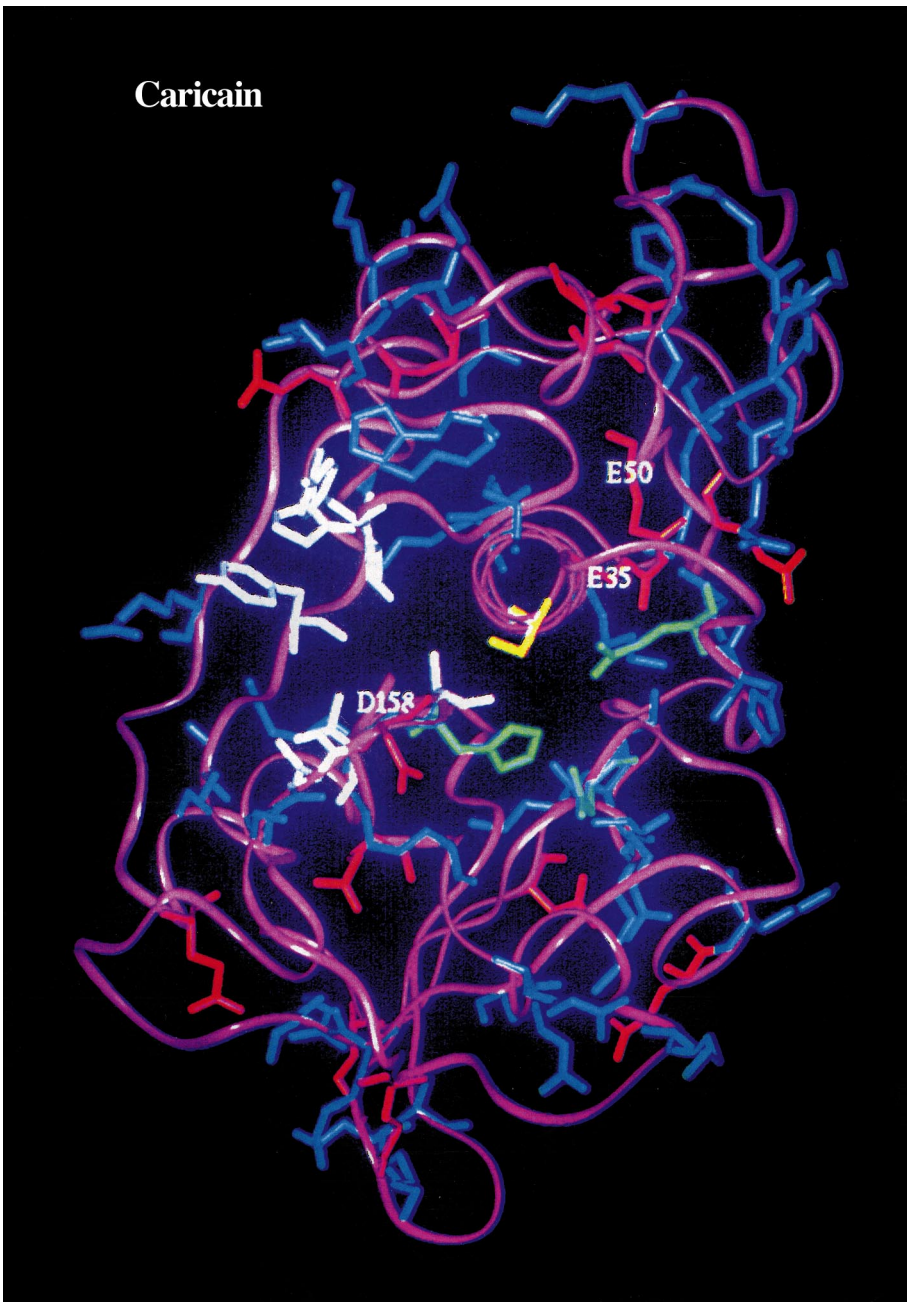

Fig. 5. A view of the cysteine proteinase caricain showing the active site cleft running from left to right between D158 and E35. Active site residues H159, Q19 are in green and C25 is yellow. Positively charged residues are blue and negatively charged residues red. The cluster of white residues on the left of the molecule delineates the hydrophobic binding site which is the S_2 subsite.

Although we did not measure the K_m at acidic pH values we hypothesise that the improved K_m of the double mutant may well suggest that the binding in the region of enhanced activity (pH 5–6) is also enhanced.

4. Discussion

It was suggested [5,15] that E50 could interact with the transition state by −52.7 kcal/mol and enhance the k_{cat}. The distance between the centroids of the S_γ and the $N_{\delta 1}$ of the ion pair and the centroids of the oxygen atoms of the carboxylate group of E50 is 12.54 Å. Pinitglang et al. [15] have discussed the mechanism of caricain/papain in some detail and propose that when the substrate is non-ionic and contains F as the occupant of the hydrophobic P2 site and G in the P1 position, an electronic switch could be present which is identified as E50. However, this did not take into account earlier evidence using the pEFLNA substrate, which is neutral in the region of the P1 site [6], which showed that the K_m was the same at pH 4.0 as at pH 6.0 but at pH 4.5–5.5 it was much less efficient. This could be taken as indicating that the ionisation of a residue with a pK_a of around 4.0 seems to disrupt binding of a neutral substrate. However, the identification of E50 as that residue should be viewed with some caution, even though E50 and its associated charge network (R17 hydrogen bonds to E35 and R83 to E50) is in a predominantly hydro-

phobic environment [15], as the binding site for a hydrophobic side chain (the S_2 subsite) occurs at such a distance from E50 that direct interaction seems unlikely (Fig. 5). Without crystallographic evidence the possible change in the position of binding of a hydrophobic side chain, following some conformational adjustment of E50A, cannot, of course, be ruled out. It appears, therefore, that from the results presented here we have shown that the removal of charge at position 50 in caricain reduces the K_m and so improves the affinity of neutral substrates. The environment surrounding the E50 charge network is hydrophobic and addition of alanine will reduce the electrostatic field, increase hydrophobicity and could increase the attraction of hydrophobic substrates to the S_2 pocket. Despite the fact that in D158E a slight change in position of the glutamic acid [2,3], coupled with its slightly increased hydrogen bonding with main-chain residues 136 and 137, apparently reduces the interaction with the substrate, in mutant D158E, E50A the K_m is improved by 3.5-fold when compared to D158E, and is also better than the wild type, but the k_{cat} is reduced as the interaction with the TS is reduced. However, overall the D158E, E50A mutant is a much more efficient enzyme than the D158E mutant vs neutral substrates.

The activity of both E50A mutants, compared to wild type and the single mutant, is improved in the pH range 4–5, exactly where E50 is calculated to ionise (3.85 ± 0.03 [15]); indeed, Sumner et al. [6] showed a sharp change in K_m values using a neutral substrate at pH 4.2. The combination of the two mutations clearly increases the binding to a greater extent than found even in the wild type at the pH optima. The double mutant becomes an improved enzyme against this neutral substrate when compared to D158E and the single mutant is superior to the wild type at pH values of 4–6. The pK_a calculation still indicates three hydronations controlling activity in the acid region. Whether these represent mirage pK_as or ionisations of E35, 57 or 135 remains to be determined. The hypothesis that an electrostatic switch is necessary to permit the thiolate and imidazolium components to provide catalysis is not disproved but clearly E50 is acting as a negative switch at some pH values when the substrates are neutral. It appears to be doing this by acting as a negative determinate of binding at pH values above the normal pH of ionisation (4.0). Below this value neutral substrates bind well but above the ionisation K_m is less good until close to the pH optima when it again reaches the same value as at pH 3.8 [6]. This explains the resultant k_{cat}/K_m curve vs pH and its dramatic change in the E50A mutants. Clearly the removal of charge at position 50 does not prevent catalytic competence in the ion pair and, indeed, it improves binding of hydrophobic substrates. It may be of interest to see if the mutant D158A, E50A has increased activity over that found in [12] for D158A, but the basic hypothesis that the ion pair (thiolate, imidazolium) in the active site does not have catalytic competence without other charged residues present [3,15] is now called into question.

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