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Importance of Hydrogen-Bonding Interactions Involving the Side Chain of Asp158 in the Catalytic Mechanism of Papain[†]

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Received January 15, 1991; Revised Manuscript Received March 15, 1991

ABSTRACT: In a previous study, it was shown that replacing Asp158 in papain by Asn had little effect on activity and that the negatively charged carboxylate of Asp158 does not significantly stabilize the active site thiolate–imidazolium ion pair of papain (Ménard et al., 1990). In this paper, we report the kinetic characterization of three more mutants at this position: Asp158Gly, Asp158Ala, and Asp158Glu. From the pH–activity profiles of these and other mutants of papain, it has been possible to develop a model that enables us to dissect out the contribution of the various mutations toward (i) intrinsic activity, (ii) ion pair stability, and (iii) the electrostatic potential at the active site. Results obtained with mutants that place either Gly or Ala at position 158 indicate that the hydrogen bonds involving the side chain of Asp158 in wild-type papain are indirectly important for enzyme activity. When CBZ-Phe-Arg-MCA is used as a substrate, the $(k_{cat}/K_M)_{obs}$ values at pH 6.5 are 3650 and 494 $M^{-1}\ s^{-1}$ for Asp158Gly and Asp158Ala, respectively, as compared to 119 000 $M^{-1}\ s^{-1}$ for papain. Results with the Asp158Glu mutant suggest that the side chain of Glu moves closer to the active site and cannot form hydrogen bonds similar to those involving Asp158 in papain. From the four mutations introduced at position 158 in papain, we can conclude that it is not the charge but the hydrogen-bonding interactions involving the side chain of Asp158 that contribute the most to the stabilization of the thiolate–imidazolium ion pair in papain. However, the charge and the hydrogen bonds of Asp158 both contribute to the intrinsic activity of the enzyme.

An understanding of the mode of action of cysteine proteases is of interest for many reasons. Cysteine proteases are members of a class of enzymes that are widely distributed among living organisms and participate in a number of physiological processes in plants, bacteria, and animals (Brocklehurst et al., 1987; Baker & Drenth, 1987). There is a great deal of evidence that implicates cysteine proteases in various disease states. In particular, lysosomal cathepsins are believed to be involved in several pathological conditions such as muscular dystrophy, heart diseases, inflammatory diseases, and tumor invasiveness (Kar & Pearson, 1977; Poole et al., 1978, 1980; Mort et al., 1984; Sloane & Honn, 1984; Rich, 1986). These enzymes form a family of homologous proteins with comparable structures (Kamphuis et al., 1985) and functions, and the information obtained from a given enzyme can be very useful in understanding the behavior of related cysteine proteases. The most widely studied member of this group is the plant protease papain, and it is taken as the prototypic member of this class of enzymes.

The structure of papain has been solved at 1.65-Å resolution and is available (Kamphuis et al., 1984), and the general

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0006-2960/91/0430-5531$02.50/0 © 1991 American Chemical Society

catalytic mechanism is well understood [see Brocklehurst et al. (1987) and Baker and Drenth (1987) for reviews]. Even though the catalytic mechanism of papain has been extensively characterized, there is still some uncertainty about the role of specific residues of the enzymes. The state of ionization of the thiol and imidazole groups at the active site has been the subject of debate, but it is now generally accepted that the thiolate–imidazolium ion pair is involved in the catalytic action of papain (Polgar, 1974; Lewis et al., 1976, 1981; Sluyterman & Wijdenes, 1976). However, the relative significance of the various factors contributing to the stability of this ion pair remains unknown. It has been proposed on theoretical grounds that the presence of the negatively charged side chain of an aspartic acid at position 158 in papain is essential for the stabilization of the ion pair and for catalysis to occur (Dijkman et al., 1989; Rullman et al., 1989). There have also been many other roles assigned to that aspartic acid, mainly on the basis of the fact that this is the only charged side chain within 10 Å of the active site of papain [e.g., Williams and Whitaker (1968), Löffler and Schneider (1974), Bendall and Lowe (1976), Angelides and Fink (1978), Lewis et al. (1978), Zannis and Kirsch (1978), Brocklehurst et al. (1983, 1984), and Salih et al. (1987)]. Comparison of amino acid sequences of related proteases shows, however, that the corresponding residues in cathepsin B's and cathepsin H's from many sources are a glycine and an asparagine, respectively. Moreover, the carboxylate side chain of Asp158 in papain is oriented away from the active site cysteine–histidine pair as shown by the 3-D structure of Kamphuis et al. (1984). These observations argue against an essential role for this aspartic acid.

With the availability of a synthetic gene coding for the papain precursor (Vernet et al., 1989) and its expression in a baculovirus–insect cell system (Vernet et al., 1990), it is now possible to obtain more precise information on the role of particular amino acid residues in papain and, by analogy, in related enzymes. Because of its proximity to the catalytic site thiolate–imidazolium ion pair and the fact that it has had a number of roles assigned to it, aspartic acid 158 is one of the best initial candidates for site-directed mutagenesis studies. Groups remote from the active site can play important roles in the mechanism of an enzyme, but they are more difficult to identify. By analogy with cathepsins B and H, the first changes to make are to replace the aspartic acid by glycine and asparagine, respectively. Many questions regarding the possible function of Asp158 in papain were answered recently with the kinetic characterization of the Asp158Asn variant of papain (Ménard et al., 1990). Replacement of the aspartic acid by an asparagine, while removing the negative charge on the side chain, causes minimal change in the van der Walls surface because of the similar volume and shape of the two amino acids. In addition, the hydrogen-bonding network involving the side chain of Asp158 and the amide groups of His159 and Ala136 as well as a water molecule can be maintained in the Asp158Asn mutant (Ménard et al., 1990). That study clearly demonstrated that Asp158 is not an essential residue for catalysis by papain since only a 6-fold decrease in k_{cat}/K_M was observed upon substitution of the aspartic acid by an asparagine. It was also shown that the side-chain carboxyl group of Asp158 is not one of the groups with pK_a's of approximately 4 that modulate the pH–activity profile of papain in the acid limb (Lewis et al., 1978). In addition, the negatively charged side chain of Asp158 does not significantly stabilize the active site thiolate–imidazolium ion pair, but its presence does influence the pK_a's associated with ion pair formation through electrostatic interaction.

The hydrogen-bonding capabilities of the Asp and Asn side chains in wild-type papain and Asp158Asn may also contribute to the catalytic mechanism of these enzymes. To investigate this possibility, we have replaced Asp158 by glycine, alanine, and glutamic acid residues, and we report here the kinetic characterization of these mutant enzymes. The side chains in the Asp158Gly and Asp158Ala mutants cannot form hydrogen bonds with the main-chain amide groups of His159 and Ala136 as found in papain, while the Asp158Glu variant has been produced to investigate the tolerance of the enzyme to the addition of one methylene group in this region of the protein. Because the active form of papain, which contains the thiolate–imidazolium ion pair, is in equilibrium with a nonactive form containing the uncharged thiolate–imidazole pair, any mutation that influences the stability of the ion pair form will consequently affect the activity of the enzyme since it will produce changes in the proportion of the enzyme that is active. A mutation can therefore have a direct effect on the intrinsic activity of papain or an indirect effect through modification of the Cys25–His159 ion pair stability. From the four mutations introduced at position 158 of papain (Asp → Asn, Gly, Ala, and Glu), the contributions of electrostatic interactions and of the hydrogen-bonding network of the Asp158 side chain to the stability of the thiolate–imidazolium ion pair and to the overall activity of the enzyme can be evaluated.

MATERIALS AND METHODS

Lentil lectin Sepharose 4B and thiopropyl-Sepharose were obtained from Pharmacia. The substrate CBZ-Phe-Arg-MCA[1] and the inhibitor E-64 were purchased from IAF Biochem International Inc., Laval, Québec. (Dns)-Phe-Arg-Phe(NO_2)-Leu was a generous gift from Dr. John Mort of the Shriners Hospital, Montréal, Québec. Papain was purchased from Sigma Chemical Co. and was purified and activated as described previously (Ménard et al., 1990).

Site-Directed Mutagenesis. IpDC127 single-stranded DNA has been used as a template for in vitro side-directed mutagenesis according to a set of procedures described elsewhere (Vernet et al., 1990). The mutations were designed not only to alter the encoded protein but also to modify restriction sites to facilitate diagnosis of the mutants after their introduction. The oligodeoxyribonucleotide used for the construction of the Asp158Glu mutant, 5′-GTAACAAGGTGGAGCATGCTGTTG-3′, simultaneously creates a *Sph*I restriction site and destroys a *Sal*I restriction site. The Asp158Gly mutation was generated with the oligodeoxyribonucleotide, 5′-TAACAAGGTCGGACATGCTGTTGC-3′, which destroys a *Sal*I restriction site. The Asp158Ala mutation was constructed with the oligodeoxyribonucleotide 5′-TAACAAGGTCGCACATGCTGTTGC-3′, which also destroys a *Sal*I restriction site. The resulting plasmids are respectively called IpDC135, IpDC145, and IpDC157. The genes for the papain precursor mutants were completely sequenced, and no alterations other than those designed were found. Recombinant baculoviruses were constructed by cell-mediated homologous recombination between plasmid IpDC135, IpDC145, or IpDC157 and the *Autographa californica* nuclear polyhedrosis virus genome (Summers & Smith, 1987). The resulting recombinant ba-

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[1] Abbreviations: CBZ-Phe-Arg-MCA, carbobenzoxy-L-phenylalanyl-(7-amino-4-methylcoumarinyl)-L-arginine; E-64, 1-[[(L-*trans*-epoxysuccinyl)-L-leucyl]amino]-4-guanidinobutane; MCA, 7-amino-4-methylcoumarin; (Dns)-Phe-Arg-Phe(NO_2)-Leu, 5-(dimethylamino)-naphthalene-1-sulfonyl-L-phenylalanyl-L-argininyl-*p*-nitrophenylalanyl-L-leucine.

culoviruses are designated respectively as Ac[preproP D158E], Ac[preproP D158G], and Ac[preproP D158A]. The presence of the designed mutations within the recombinant baculoviruses was confirmed by restriction analysis of viral DNA fragments amplified by the polymerase chain reaction as described elsewhere (Tessier et al., 1991). For the production of the recombinant proteins, *Spodoptera frugiperda* Sf9 cells were grown and infected in serum-supplemented medium. The cells were centrifuged and washed 2 h postinfection in conditions described previously (Vernet et al., 1990) except that the osmolarity of the Grace's medium was maintained at 380 mosm by adding 2 mL of sterile 80% glycerol per liter of medium. The secreted product was harvested 48–74 h postinfection. Site-directed mutagenesis of the papain gene to give a Ser176Ala mutant has been described elsewhere (Ménard et al., 1991).

Purification of Recombinant Papain. The Asp158Gly, Asp158Ala, and Asp158Glu mutants were purified by a combination of affinity chromatography and covalent chromatography. The culture medium was concentrated approximately 10-fold by ultrafiltration using an Amicon Spiral Membrane Cartridge type S1Y10. In a first step, the secreted recombinant protein was subjected to affinity chromatography on lentil lectin Sepharose 4B. The concentrated medium was loaded onto the lentil lectin column (100 mm × 10 mm i.d.), which had previously been equilibrated with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 M NaCl (buffer A). The column was washed with 10 bed volumes of buffer A, and the bound proteins were eluted with 10 bed volumes of the same buffer containing 0.2 M methyl mannopyranoside. The precursor protein that eluted from the lentil lectin column was activated in vitro to the mature enzyme (Vernet et al., 1990). Cysteine was added to the eluted fraction to a final concentration of 20 mM, and the pH was adjusted to 4.0 by the addition of 0.1 M acetate buffer. The mixture was incubated at 50 °C for 30–60 min to accelerate the processing of the precursor. After an ammonium sulfate precipitation of the supernatant, the recombinant protein was further purified by covalent chromatography on a thiopropyl-Sepharose column as described previously (Ménard et al., 1990). Purification of the Ser176Ala mutant was as described in a previous paper (Ménard et al., 1991).

Kinetic Measurements. The kinetic experiments were performed as described previously (Ménard et al., 1990). The concentration of the papain variants Asp158Gly, Asp158Ala, and Asp158Glu was determined by active site titration with E-64 (Barrett & Kirschke, 1981). Hydrolysis of the substrate CBZ-Phe-Arg-MCA was monitored by measuring the fluorescence of the MCA product. Kinetic parameters at pH 6.5 were determined from initial rate measurements at various substrate concentrations under the following conditions: 50 mM phosphate buffer, 0.2 M NaCl, 5 mM EDTA, and 20% CH_3CN. Kinetic parameters were obtained by linear regression of the data to plots of s/v vs s (Hanes plots). To evaluate the influence of pH on activity, the initial rates were measured at substrate concentrations much lower than the K_M[2] value for the enzyme–substrate system under study. The value of $(k_{cat}/K_M)_{obs}$ is then obtained from the relation $v_i = e_0 s (k_{cat}/K_M)_{obs}$, where e_0 is the enzyme concentration. Experimental conditions for pH–activity studies were the same as for studies at pH 6.5, except that the phosphate buffer was replaced by sodium citrate for experiments at pH 3.0–5.7 and by sodium borate for experiments at pH 7.9–10.0. The pH–activity data were fitted to the model described under Results. (Dns)-Phe-Arg-Phe(NO_2)-Leu hydrolysis was monitored by the increase in fluorescence of the dansyl group produced by the cleavage of the Arg–Phe(NO_2) bond as described by Pohl et al. (1987). The excitation wavelength was 350 nm, and emission was detected at 535 nm.

Table I: Kinetic Parameters for CBZ-Phe-Arg-MCA Hydrolysis[a]

	k_{cat} (s^{-1})	K_M (mM)	$(k_{cat}/K_M)_{obs}$ (M^{-1} s^{-1})
papain[b]	52 ± 6	0.42 ± 0.07	1.19 ± 0.18 × 10^5
Asp158Gly	4.2 ± 1.0	1.3 ± 0.5	3.65 ± 1.78 × 10^3
Asp158Ala	0.46 ± 0.08	0.92 ± 0.07	4.94 ± 0.49 × 10^2
Asp158Glu	7.8 ± 4.1	0.94 ± 0.33	8.35 ± 3.48 × 10^3
Asp158Asn[b]	34 ± 6	1.78 ± 0.47	1.97 ± 0.17 × 10^4

[a] Values of k_{cat}, K_M, and $(k_{cat}/K_M)_{obs}$ are from studies at pH 6.5 using various substrate concentrations. [b] From Ménard et al. (1990).

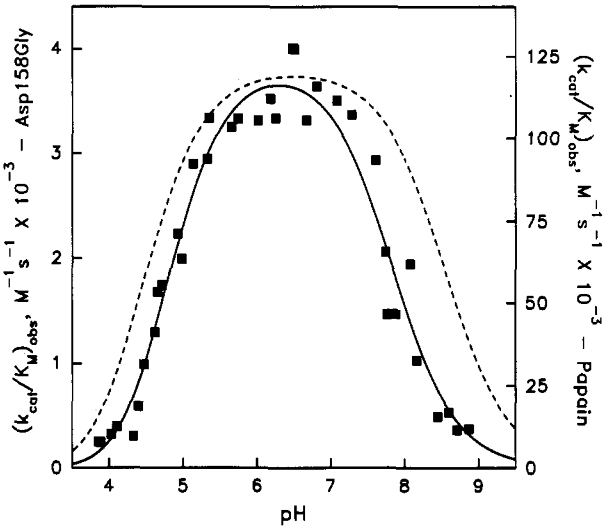

FIGURE 1: pH dependence of $(k_{cat}/K_M)_{obs}$ for the Asp158Gly mutant. At each pH, $(k_{cat}/K_M)_{obs}$ was determined by measuring the initial rate of CBZ-Phe-Arg-MCA hydrolysis and dividing by enzyme and substrate concentrations. The substrate concentration was kept constant at 0.06 mM. The solid line is the best fit to the model in Scheme I. The dashed line is the best fit for data (not shown) obtained with commercial papain.

RESULTS

Kinetic Characterization at Optimum pH. The kinetic parameters for hydrolysis of CBZ-Phe-Arg-MCA by papain and the Asp158Gly, Asp158Ala, and Asp158Glu mutants are given in Table I. The results obtained previously for the Asp158Asn variant of papain (Ménard et al., 1990) are also included in Table I for comparison. Replacing the aspartic acid residue at position 158 by a glycine causes a 33-fold decrease in $(k_{cat}/K_M)_{obs}$ with respect to the wild-type papain, while with the glutamic acid mutant a 14-fold decrease in $(k_{cat}/K_M)_{obs}$ is observed. The decrease in activity is much more important when Asp158 is replaced by an alanine since $(k_{cat}/K_M)_{obs}$ for the Asp158Ala mutant is only 4.94 × 10^2 M^{-1} s^{-1}, which corresponds to a 240-fold decrease in activity as compared to papain. In all cases, these variations are due mainly to a decrease in k_{cat}, whereas K_M is only slightly affected by the mutations.

pH–Activity Profiles. The influence of pH on $(k_{cat}/K_M)_{obs}$ for the Asp158Gly and Asp158Glu mutants is illustrated in

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[2] The following convention is used to describe kinetic data and the model: k_{cat}, K_M, and $(k_{cat}/K_M)_{obs}$ represent values of the kinetic parameters determined experimentally; $(k_{cat}/K_M)^{lim}$ and pK_i^{obs} are the limiting value of the specificity constant and the corresponding experimental pK_a's determined by nonlinear regression of the pH–$(k_{cat}/K_M)_{obs}$ data to a given equation; k_{cat}/K_M, and pK_i represent intrinsic values of the parameters describing the model shown in Scheme I.

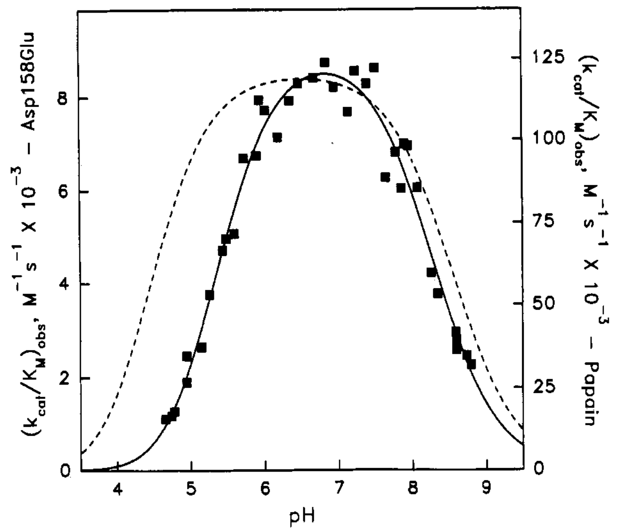
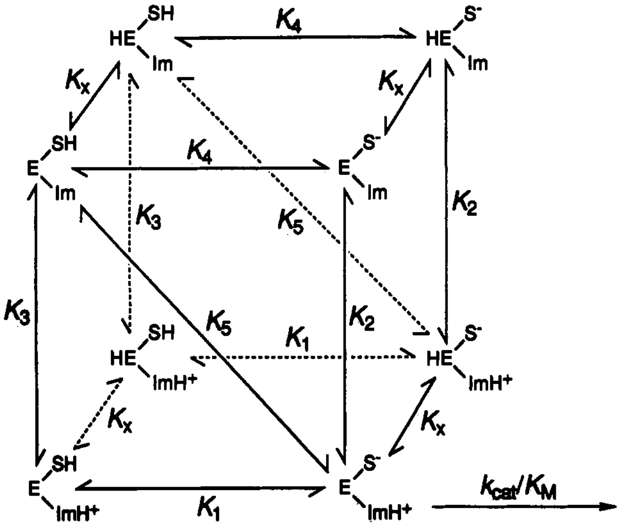


FIGURE 2: pH dependence of $(k_{cat}/K_M)_{obs}$ for the Asp158Glu mutant. Conditions are the same as described in the legend to Figure 1.

Figures 1 and 2, respectively. No reliable pH–activity profiles could be obtained with Asp158Ala due to the very low activity of this mutant. As for wild-type papain, the profiles for Asp158Gly and Asp158Glu are bell shaped with maximum activity at near neutral pH. However, these pH–activity profiles are significantly narrower than that of the wild-type enzyme. In addition, the profiles are shifted along the pH scale compared to papain, where maximum activity is obtained at pH_{max} 6.50 (Table II). For the Gly mutant, maximum activity is reached at a pH value lower than 6.50, as was the case for the Asn mutant, while pH_{max} with the Glu mutant is at 0.35 pH units higher than that of wild-type papain. The pH–activity profile of a Ser176Ala mutant, which has been characterized previously (Mênard et al., 1991), was also determined under the experimental conditions of the present study. This mutant was originally designed to investigate the role of the interdomain hydrogen bond formed between the side chains of Ser176 and Gln19, one of the hydrogen-bond donors in the putative oxyanion hole of papain. As will be discussed in the later portion of this paper, the Ser176Ala mutant can be used to approximate the (ion pair form $\rightleftharpoons$ neutral form) equilibrium constant of the active site residues in papain (Mênard et al, 1991).

Ionization constants of residues modulating activity for papain and the Asp158Gly, Asp158Glu, and Ser176Ala mutants were obtained by nonlinear regression of pH–activity data to eq 1. In addition, previously published data for the Asp158Asn mutant (Mênard et al., 1990) was reinvestigated by using this equation, and the results are given in Table II. As was previously observed for papain and the Asp158Asn and Ser176Ala mutants, the activity in the acid limb of the profiles for both the Gly158 and Glu158 derivatives of papain cannot be described by a single ionization, and at least one more ionizable group must be considered in order to fit the profiles. Whereas in papain the two pK_a's are approximately 4 (3.81 and 4.34), they are 4.35 and 4.57 for the Asp158Gly mutant and 4.32 and 5.34 for Asp158Glu. A question that remains unanswered in papain-catalyzed hydrolyses is the nature of the group defining the second pK_a-modulating activity in the low pH region and its assignment to either pK_1^{obs} or pK_x^{obs}. The nonlinear regression procedure used in this study will always attribute the lowest of the two pK_a's in the acid region to pK_x^{obs} regardless of the nature of the group to which it corresponds, and results of the regression cannot be used to assign one of these two pK_a's to Cys25. In the present study, we have considered that pK_1^{obs} represents deprotonation of the active site Cys25 on the basis of the observation of the effects of various mutations on pK_1^{obs} and pK_x^{obs}. It can be seen in Table II that, within experimental error, pK_x^{obs} remains fairly constant for all the mutants produced, while variations in pK_1^{obs} are much more important (especially in the case of Asp158Glu). It is very unlikely that the broad range of mutations at position 158 in addition to the mutation at position 176 would have relatively large effects on ionization of the unknown group while having a negligible effect on ionization of the active site Cys25, which is believed to be very sensitive to environmental effects. Therefore, pK_1^{obs} corresponds most likely to deprotonation of Cys25. There is at present no simple experiment that would allow us to assign without any doubt pK_1^{obs} to Cys25, but further site-directed mutagenesis studies might help in identifying the second ionizable residue and solve this problem.

Scheme I



Stability of the Thiolate–Imidazolium Form of Papain. To understand the behavior of papain, one has to consider at least four enzyme species corresponding to various protonation states of the active site residues Cys25 and His159 (Mênard et al., 1990). In addition, since at least one other residue on the enzyme with a pK_a of ca. 4 has been shown to influence the activity of the enzyme, the ionization state of this residue has to be considered, which leads to a total of eight possible enzyme species, as depicted in the model shown in Scheme I. K_1 to K_4 are the ionization constants of the two active site residues while K_x represents the ionization constant of the unknown residue and is considered here to be not significantly affected by the protonation state of Cys25 and His159. K_5 is an equilibrium constant for the conversion of the neutral form of the active site of papain to the thiolate–imidazolium ion pair form. For this model, pH–activity profiles can be described by

$$(k_{cat}/K_M)_{obs} = \frac{(k_{cat}/K_M)^{lim}}{\left(\frac{[H^+]}{K_1^{obs}} + 1 + \frac{K_2^{obs}}{[H^+]}\right)\cdot\left(1 + \frac{[H^+]}{K_x^{obs}}\right)} \quad (1)$$

where $K_1^{obs} = K_1/[K_5/(K_5 + 1)]$, $K_2^{obs} = K_2\cdot[K_5/(K_5 + 1)]$ and $K_x^{obs} = K_x$. In addition, for the wild-type enzyme

$$(k_{cat}/K_M)^{lim}_{WT} = (k_{cat}/K_M)_{WT}\cdot\left(\frac{K_5}{K_5 + 1}\right) \quad (2)$$

where $(k_{cat}/K_M)^{lim}_{WT}$ is the limiting value of $(k_{cat}/K_M)_{obs}$ in eq

Table II: Analysis of pH–Activity Profiles and Determination of r, $rK_5/(rK_5 + 1)$, and f for Hydrolysis of CBZ-Phe-Arg-MCA by Wild-Type and Mutant Papain[a]

	pK_x^{obs}	pK_1^{obs}	pK_2^{obs}	$(k_{cat}/K_M)^{lim}$ ($M^{-1}\ s^{-1}$)	pH_{max}	$\Delta\Delta pK$	r	$rK_5/(rK_5 + 1)$	f
papain[b]	3.81 ± 0.38	4.34 ± 0.18	8.54 ± 0.10	$1.21 \pm 0.18 \times 10^5$	6.50 ± 0.10		1	0.82	1
Asp158Gly	4.35 ± 0.59	4.57 ± 0.56	7.89 ± 0.08	$3.87 \pm 1.88 \times 10^3$	6.33 ± 0.28	−0.89	0.21	0.49	0.05
Asp158Glu	4.32 ± 0.36	5.34 ± 0.09	8.32 ± 0.06	$9.12 \pm 3.80 \times 10^3$	6.85 ± 0.05	−1.23	0.07	0.24	0.25
Asp158Asn[b]	3.76 ± 0.09	4.04 ± 0.06	8.16 [illegible] 0.03	$2.02 \pm 0.17 \times 10^4$	6.20 ± 0.03	−0.09	0.88	0.80	0.17
Ser176Ala	4.10 ± 0.36	4.80 ± 0.25	8.27 ± 0.05	$8.46 \pm 0.38 \times 10^4$	6.57 ± 0.13	−0.74	0.30	0.57	1

[a] Values of pK_a's and of $(k_{cat}/K_M)^{lim}$ were obtained by nonlinear regression of pH–activity data to eq 1; pH_{max}, $\Delta\Delta pK$, r, $rK_5/(rK_5 + 1)$, and f were determined as described in the text, by using $K_5 = 4.4$ in the calculations. [b] Analysis of previously published data (Ménard et al., 1990).

1 and $(k_{cat}/K_M)_{WT}$ is the intrinsic value of k_{cat}/K_M in the model in Scheme I for the wild-type enzyme. From eq 2, $(k_{cat}/K_M)_{WT}^{lim}$ will decrease if the thiolate–imidazolium form of papain is destabilized and increase with an increase in stability of the ion pair. The experimentally determined values of pK_1^{obs} and pK_2^{obs} will also be affected by variations in K_5, while pK_x^{obs} should be independent of the stability of the ion pair. With the relations $K_1 = K_3 \cdot K_5$ and $K_2 = K_4/K_5$ from the model in Scheme I, the width of a pH–activity profile ($pK_2^{obs} - pK_1^{obs}$) can be described by

$$(pK_2^{obs} - pK_1^{obs}) = (pK_4 - pK_3) + 2 \cdot \log (K_5 + 1) \quad (3)$$

From eq 3 we can see that any additional factor stabilizing the ion pair form of papain will cause an increase in ($pK_2^{obs} - pK_1^{obs}$), while a decrease in width will be observed when the ion pair is destabilized. If $\Delta\Delta pK$ represents the variation in width of a pH–activity profile on proceeding from papain to a mutant, i.e., $\Delta\Delta pK = (pK_2^{obs} - pK_1^{obs})_{mut} - (pK_2^{obs} - pK_1^{obs})_{WT}$ and if we assume that $pK_4 - pK_3$, the difference in pK_a's for the ionization of Cys25 and His159 in absence of factors stabilizing the ion pair, is not affected by the mutation, then from eq 3 we can write that

$$\Delta\Delta pK = 2 \cdot \log \left(\frac{rK_5 + 1}{K_5 + 1} \right) \quad (4)$$

where $r = K_{5mut}/K_5$ with K_{5mut} being the equilibrium constant for a mutant of papain. The value of r will therefore represent the effect of a mutation on the stability of the ion pair.

The specificity constant for a papain mutant can be written as

$$(k_{cat}/K_M)_{mut}^{lim} = (k_{cat}/K_M)_{WT} \cdot f \cdot \left(\frac{rK_5}{rK_5 + 1} \right) \quad (5)$$

with $f = (k_{cat}/K_M)_{mut}/(k_{cat}/K_M)_{WT}$. From this equation it can be seen that a perturbation of the equilibrium between the neutral and ion pair forms of papain will affect $(k_{cat}/K_M)_{obs}$ by a factor of $[rK_5/(rK_5 + 1)]$, while f takes into consideration the influence of the mutation on other aspects of the mechanism (i.e., binding, reactivity, etc.). When eqs 4 and 5 are combined

$$K_5 = \frac{10^{-(\Delta\Delta pK/2)} - 1}{1 - \alpha/f} \quad (6)$$

where $\alpha = (k_{cat}/K_M)_{mut}^{lim}/(k_{cat}/K_M)_{WT}^{lim}$. From this equation it can be seen that for any fixed value of $\Delta\Delta pK$, K_5 approaches a minimum value when $\alpha/f \rightarrow 0$, and from the largest $\Delta\Delta pK$ in Table II an overall minimum of $K_5 = 3.1$ can be calculated.

If a mutation was to influence only the stability of the ion pair, i.e., $f = 1$ in eqs 5 and 6, then the value of K_5 could be obtained from the $\Delta\Delta pK$ and the $(k_{cat}/K_M)^{lim}$ values of the mutant. In the case of the Ser176Ala mutant described in a previous paper (Ménard et al., 1991), it has been suggested that the mutation caused a destabilization of the ion pair form of papain. Assuming that the effect of the Ser176Ala mutation is due exclusively to a perturbation of the ion pair stability, the value of K_5 for papain and of r for the Ser176Ala mutant can be evaluated from eq 6 (with $f = 1$) and eq 4, respectively. With the data given in Table II, a value of 4.4 was found for K_5. This value compares well to those listed in the literature, ranging from 2 to 12 (Lewis et al., 1976; Creighton & Schamp, 1980), which gives some support to our interpretation of the data. As shown in Table II, the effect of the mutation on the ion pair stability is characterized by an r value of 0.30 for the Ser176Ala mutant.

Table III: Intrinsic pK_a's for the Ionization of Active Site Residues Cys25 and His159 According to the Model in Scheme I[a]

	pK_1	pK_2	pK_3	pK_4
papain	4.43	8.46	5.08	7.81
Asp158Gly	4.88	7.57	4.86	7.60
Asp158Glu	5.96	7.70	5.46	8.20
Asp158Asn	4.14	8.06	4.74	7.47
Ser176Ala	5.05	8.02	5.17	7.90

[a] Calculated from experimental pK_a's in Table II and with $K_5 = 4.4$ as described in the text.

With the value of $K_5 = 4.4$, the data from pH–activity profiles can now be analyzed with eqs 4 and 5 to determine values of r, $rK_5/(rK_5 + 1)$, and f for any mutant of papain and evaluate the effect of the mutation on the stability of its ion pair and on the intrinsic reactivity of the enzyme. Results for the Asp158Asn, Asp158Gly, and Asp158Glu mutants are given in Table II. As discussed in a previous paper (Ménard et al., 1990), the replacement of Asp158 by an asparagine has little influence on ion pair stability ($r = 0.88$), and the major influence of the mutation is on the intrinsic activity of the enzyme, which is 6-fold lower than in the wild-type ($f = 0.17$). However, if a glycine or a glutamic acid residue is introduced at position 158 the effect on the ion pair stability is much more significant, as evidenced by the narrower pH–activity profiles ($\Delta\Delta pK = 0.89$ and -1.23 pH units, giving rise to r values of 0.21 and 0.07 for Asp158Gly and Asp158Glu, respectively). Part of the decrease in k_{cat}/K_M observed for these mutants is therefore attributable to a destabilization of the thiolate–imidazolium ion pair. The value of f for the Asp158Gly variant is 0.05 indicating that the intrinsic activity is 20-fold lower than in wild-type papain. With the Asp158Glu mutant, $f = 0.25$ and the mutant enzyme is only 4 times less active than the wild-type. For both of these mutants, the actual decrease in intrinsic activity is therefore less important than is suggested by the measurement of overall activity. With the Asp158Asn mutant, the observed and intrinsic activities are approximately the same since the mutation has no significant effect on ion pair stability.

The intrinsic pK_a's of the model in Scheme I for deprotonation of the active site Cys25 and His159 residues can also be obtained when K_5 is known by using the relationships $K_1 = K_1^{obs} \cdot [K_5/(K_5 + 1)]$, $K_2 = K_2^{obs}/[K_5/(K_5 + 1)]$, $K_3 = K_1/K_5$, and $K_4 = K_2 \cdot K_5$. The results are given in Table III.

Assay for Exopeptidase Activity. To check if the mutant enzymes can degrade synthetic substrates by an exopeptidase

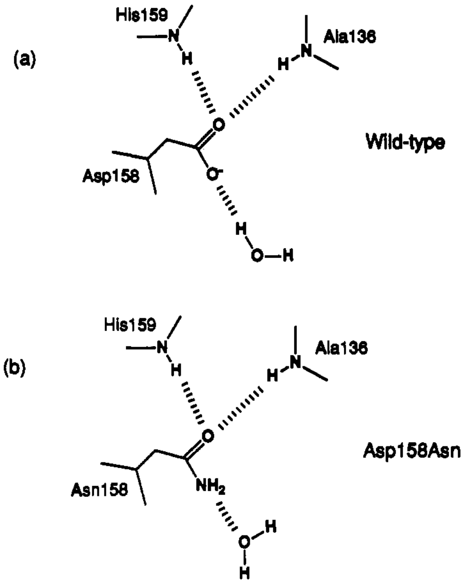


FIGURE 3: Hydrogen bonds formed by the side chain of residue 158 in papain: (a) Wild-type papain, based on the 3-D structure of Kamphuis et al. (1984); (b) Postulated hydrogen bonds in the Asp158Asn mutant.

mechanism, the enzymes were assayed by using (Dns)-Phe-Arg-Phe(NO_2)-Leu to check for dipeptidyl carboxypeptidase activity (Pohl et al., 1987). The reactions were carried out at various pH values ranging from 4.0 to 6.5, and no significant hydrolysis of the substrate was detected with wild-type or mutant papain.

DISCUSSION

The importance of Asp158 in the catalytic mechanism of papain has been debated for many years. Most of the discussion centered around the fact that the side chain of Asp158 bears the only charge within 10 Å of the thiolate–imidazolium ion pair, which constitutes the active form the enzyme. However, the hydrogen-bonding network illustrated in Figure 3a involving the Asp158 side-chain carboxylate oxygens ($O_{\delta1}$ and $O_{\delta2}$) with residues Ala136 ($O_{\delta1}$–NH distance = 2.74 Å) and His159 ($O_{\delta1}$–NH distance = 3.03 Å) and with a water molecule ($O_{\delta2}$–W38 distance = 2.60 Å) has been overlooked. In a previous study, Ménard et al. (1990) showed that eliminating the charge by replacing Asp158 by an asparagine had little effect on the activity of the enzyme. The mutation of an aspartic acid to an asparagine that is isosteric is expected to cause minimal conformational changes, if any, while the side chain has the possibility to maintain the hydrogen-bonding interactions present in the wild-type enzyme (see Figure 3b). Thus, the effects observed by replacing Asp158 by an asparagine residue can be attributed mainly to removal of the negative charge in the vicinity of the active site. When Asp158 is replaced by a glycine or an alanine residue, the hydrogen-bonding network cannot be maintained; and these mutations lead to large changes in catalytic parameters and in pH–activity profiles of the enzyme (see Table I and Figures 1 and 2), presumably due to an increased flexibility of this portion of the enzyme's structure.

The electrostatic effect of the negatively charged side chain of Asp158 on the pK_a's associated with ion pair formation suggested in the previous study (Ménard et al., 1990) is supported by the results of the present work. In mutants where the possibility of side-chain ionization is removed (i.e., Asp158Asn and Asp158Gly), the pH–activity profiles are shifted to lower pH as indicated by the decrease in pH_{max} (Table II). With a glutamic acid at position 158, the pH–activity profile is shifted in the opposite direction i.e., pH_{max} increases by 0.35 pH units. Since the side chain of a glutamic acid is longer than that of an aspartic acid, it is likely that the negative charge of Glu158 is located closer to the active site ion pair in the Asp158Glu mutant than the negative charge of Asp158 in wild-type papain. From electrostatic considerations, if a charge moves closer to the active site ion pair (up to a certain limit), it is predicted to cause an effect opposite to that of either moving the charge away from the active site or removing it completely by mutation. A more detailed analysis of electrostatic interactions in the Asp158Glu mutant awaits the availability of a 3-D structure for this mutant enzyme.

The significance of results obtained with the four mutants at position 158 is evaluated by considering the model in Scheme I, which takes into account the possible ionization pathways of residues on the enzyme that affect the activity upon deprotonation. This model considers nonsequential ionizations and is more accurate than the sequential ionizations model used in our previous publication (Ménard et al., 1990). The similarity of the pK_a's obtained for papain and Asp158Asn with the two models indicates, however, that the sequential ionizations model is a good approximation of the more complex system presented in this paper.

A central point of the present analysis is the relation that was established between the width of a pH–activity profile and the stability of the ion pair form of papain. The assumption that the active form of papain consists of a thiolate–imidazolium ion pair in equilibrium with the neutral form of the active site residues is now generally accepted as experimental evidence for its existence accumulates (Polgar, 1974; Lewis et al., 1976; Sluyterman & Wijdenes, 1976; Lewis et al., 1981). It is a necessary consequence of the model in Scheme I that the experimental values of pK_a^{obs} and $(k_{cat}/K_M)_{obs}$ will be affected by changes in the stability of the ion pair, i.e., changes in K_5. Mutations can therefore affect activity through perturbation of the ion pair equilibrium or through a direct effect on intrinsic activity. Equations 4 and 5 have been used to dissect out the contribution of variation in ion pair stability and the intrinsic activity of each mutant. The validity of these equations depends on the assumption that $pK_4 - pK_3$ in the model in Scheme I is not affected by the mutation being considered. This assumption can reasonably be made if one considers that pK_3 and pK_4 represent the ionization constants of the active site residues His159 and Cys25 in absence of factors that contribute to the stabilization of the ion pair form of papain. For this reason, they are expected to be much less sensitive to environmental effects than the corresponding pK_1 and pK_2 values that mutually interact and should be very dependent on small modifications of the environment (Lewis et al., 1981; Pickersgill, 1988). Given that eq 4 requires only that $pK_4 - pK_3$ be constant, it is important to note that electrostatic factors have been shown to affect the pK_a values of the active site Cys25 and His159 in the same direction (Ménard et al., 1990). Thus, the value of $pK_4 - pK_3$ will not vary significantly with the electrostatic environment unless the magnitude of the electrostatic effect is very different for the two active site residues. The intrinsic values of pK_3 and pK_4 listed in Table III vary from one enzyme to another due to electrostatic interactions that cannot be evaluated quantitatively. It must be noted, however, that these pK_a's are very similar for enzymes where the electrostatic contribution of residue 158 should be comparable (i.e., wild-type versus Ser176Ala papain and Asp158Asn versus Asp158Gly papain).

These values of pK_3 and pK_4 are also very close to the usual range for side-chain ionization of histidine (pK_a 5–8) and cysteine (pK_a 8–11) in a protein (Fersht, 1985).

The results of the data analysis reported in Table II are also dependent on the value of K_5 used in our calculations. This value was obtained from analysis of a Ser176Ala mutation in papain, where it is considered that the only significant contribution to the decrease in activity as compared to wild-type enzyme is a destabiliztaion of the thiolate–imidazolium ion pair (Mênard et al., 1991). The validity of this assumption has been discussed previously, and it is reassuring to note that the value of $K_5 = 4.4$ obtained in this manner is in good agreement with published values determined by different experimental methodologies (Lewis et al., 1976; Creighton & Schamp, 1980). It must be noted also that qualitatively similar values to these reported in Table II are obtained if either the lower limit of $K_5 = 3.1$ determined in this study or the upper limit of $K_5 = 12$ reported in the literature (Lewis et al., 1976) is used.

From the $\Delta\Delta pK$ and r values listed in Table II, we note that the stability of the ion pair form of papain is greatly affected by the Asp158 to Gly mutation. It was shown previously that this is not a result of removing the negative charge of the Asp158 side chain (Mênard et al., 1990); however, it could be a consequence of the absence of hydrogen-bonding interactions between the side chain of residue 158 and the main-chain amide protons of Ala136 and His159 in the Asp158Gly mutant (as compared to the Asp158Asn mutant where the ion pair stability seems to be affected only slightly, $r = 0.88$, and the hydrogen-bonding interactions are believed to be maintained, see Figure 3). It therefore seems that it is not the charge but the hydrogen-bonding network involving the side chain of Asp158 in papain that is important for stabilizing the active thiolate–imidazolium form of the enzyme. This finding is not surprising, even though it has never been considered in previous studies, since residue 158 is located next to the active site His159 and since one of the hydrogen-bonding partners involved is the main-chain amide proton of His159. Modification or removal of the Asp158 side-chain interactions could easily produce small conformational changes in the environment of the active site residues that would perturb the optimum orientation of the mutually interacting Cys25 and His159 and therefore destabilize the ion pair. The low r value obtained with the Asp158Glu mutant suggests that the side chain of a glutamic acid residue at position 158 cannot form similar hydrogen bonds as those present in wild-type papain or Asp158Asn. This is consistent with the suggestion that the carboxylate side chain in Asp158Glu is located closer to the active site. Since Asp158 in wild-type papain faces away from the active site, a different side-chain conformation is necessary in the Asp158Glu mutant for the negative charge to move closer to the thiolate–imidazolium ion pair.

The factor f is a measure of the influence of a mutation on the intrinsic activity of papain. For the Asp158Asn mutant, $f = 0.17$, which corresponds to a 6-fold decrease in intrinsic activity. As was mentioned previously, the effect of this mutation should be attributable in large part to the removal of the negative charge in the vicinity of the active site, and it is likely that this could explain the 6-fold drop in intrinsic activity. For the Asp158Gly mutant, the decrease in $(k_{cat}/K_M)_{obs}$ is much more important than with the Asp158Asn mutant (Table I). The relatively important destabilization of the ion pair with this mutant is responsible for a 2-fold decrease in activity as reflected by the value of $rK_5/(rK_5 + 1) = 0.49$ (Table II). In addition, this mutation causes a 20-fold decrease in intrinsic activity ($f = 0.05$). A large part of this decrease in intrinsic activity can be attributed once again to electrostatic factors absent when the ionizable side chain of Asp158 is removed. The absence of a side chain interacting through hydrogen bonds with the main chain of papain could explain the further decrease in activity observed for this mutant.

Data analysis for the Asp158Glu mutant indicates that ion pair destabilization causes the observed k_{cat}/K_M to decrease by a factor of 4 ($rK_5/(rK_5 + 1) = 0.24$). The f value of 0.25 is high as compared to that for Asp158Gly because it reflects the combination of the unfavorable effect from the absence of hydrogen-bonding network and the favorable effect from the presence of a negative charge on the glutamate residue on activity. It is difficult, however, to evaluate the electrostatic contribution to the activity for the Asp158Glu mutant since the exact position of the negatively charged glutamate in Asp158Glu is not known.

Results with both the Asp158Gly and Asp158Glu mutants suggest that removal of the hydrogen-bonding network involving the Asp158 side chain causes the activity to decrease in part due to the resulting destabilization of the active site ion pair. However, it also has an influence on intrinsic activity, which is lower than what is expected solely on the basis of electrostatic effects from the presence or absence of ionizable side chain at position 158. This correlates in a way with the observation that k_{cat} is the most different from wild-type papain in mutants where the hydrogen-bonding ability of the residue 158 side chain is affected. It is thus possible that the effect on intrinsic activity of removing the hydrogen-bonding network could be due to a perturbation of the rate of acylation, which could also be reflected in a decrease in k_{cat}. However, the effect on k_{cat} cannot be analyzed in a straightforward manner since the nature of the rate-limiting step is unknown for the substrate used in this study. It is interesting to note that in the crystal structures of papain–chloromethylketone complexes, the main-chain carbonyl group of Asp158 is directed toward the P_1 NH of the bound substrate analogues (Drenth et al., 1976). The formation of an hydrogen bond between these two groups could occur during acylation and would most probably be affected by replacement of Asp158 by Gly or Glu.

The mutation of Asp158 to an alanine leads to an enzyme of unusually low activity considering the result obtained with the Asp158Gly mutant. Due to the small amount of enzyme available and to its low activity, we were not able to obtain a reliable pH–activity profile for this mutant, and a detailed analysis of the influence of the mutation on ion pair stability and intrinsic activity could not be obtained. The fact that $(k_{cat}/K_M)_{obs}$ is 7-fold lower for Asp158Ala as compared to Asp158Gly does indicate, however, that the presence of an alanine at position 158 in papain is much less favorable than that of a glycine. It is possible that in the case of the glycine mutant, a water molecule could be introduced in place of the Asp158 side chain to fulfill the hydrogen-bond requirements of this region of the protein, leading to an enzyme that is more active than the Asp158Ala variant, where the CH_3 side chain could prevent the substitution of a water molecule. Determination of the 3-D structure of these mutants will be very helpful for the understanding of these variations in activity.

In the related enzyme cathepsin B, the residue corresponding to Asp158 in papain is glycine. It has been suggested that, in part, cathepsin B exhibits a dipeptidyl carboxypeptidase activity due to the absence of a negative charge at position 158, which would cause repulsion of the C-terminal carboxylate group of a peptide (Lowe & Yuthavong, 1971). However, both the Asp158Asn and Asp158Gly mutants did not display any detectable dipeptidyl carboxypeptidase activity, and this possibility can now be ruled out. Stability of the ion pair is more difficult to evaluate with cathepsin B due to the complexity of pH–activity profiles for this enzyme (Khouri et al., 1991). However, the absence of a side chain in the position corresponding to residue 158 in papain does not seem to be detrimental to the activity of cathepsin B. When the 3-D structure of cathepsin B becomes available, it will be interesting to see if the overall structure or "rigidity" of this region is maintained by neighboring amino acid substitutions that could supply a compensating change.

In summary, analyzing the influence of pH on activity for mutants at position 158 in papain has allowed us to evaluate the contribution of various factors to the activity of wild-type enzyme. Mutations where the charge on the Asp158 side chain is removed (Asp158Gly, Asp158Asn) or where the distance between the charge on the side chain and the active site residues is modified (Asp158Glu) cause shifts in pH_{max} that are consistent with electrostatic interactions between the charged side chains at position 158 and the active site residues of papain. A relation between the width of a pH–activity profile and the stability of the thiolate–imidazolium form of papain has been established. For the Asp158Gly and Asp158Glu mutants, an important narrowing of pH–activity profiles is observed, indicating that the ion pair form of the enzyme is less stable for these two mutants. This has been attributed to the fact that the side chain of Asp158 forms hydrogen bonds with the main-chain amide protons of His159 and Ala136 that cannot be maintained in the mutant enzymes. When Asp158 is replaced by an Asn, the side chain of Asn can form hydrogen bonds similar to those present in the wild-type enzyme, and there is almost no effect of the mutation on the pH–activity profile width. It therefore seems that the hydrogen-bonding network involving the side chain of Asp158 is important for ion pair stabilization in papain. The decrease in activity for the various mutants has been shown to originate from perturbation of the ion pair stability, from a contribution of electrostatic interaction and from an additional effect on intrinsic activity for mutants where the side chain cannot maintain the hydrogen-bonding network of Asp158.

ACKNOWLEDGMENTS

We thank Dr. John Mort for providing the substrate used to assay for dipeptidyl carboxypeptidase activity and France Laliberté for technical assistance.

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