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A Protein Engineering Study of the Role of Aspartate 158 in the Catalytic Mechanism of Papain[†]

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ABSTRACT: The controversy concerning the various suggested roles for the side chain of Asp158 in the active site of papain has been clarified by using site-directed mutagenesis. Both wild-type papain and an Asp158Asn variant were produced in a baculovirus-insect cell expression system, purified to homogeneity from the culture, and characterized kinetically. With CBZ-Phe-Arg-MCA as substrate, the $k_{\text{cat}}/K_{\text{M}}$ and k_{cat} values obtained for the Asp158Asn papain are 20 000 M⁻¹·s⁻¹ and 34 s⁻¹, respectively, as compared with values of 120 000 M⁻¹·s⁻¹ and 51 s⁻¹ obtained for the wild-type papain. In addition, the pH-($k_{\text{cat}}/K_{\text{M}}$) profile for the Asp158Asn enzyme is shifted relative to that for the wild-type enzyme to lower values by approximately 0.3 pH unit. This shows clearly that Asp158 is not, as previously postulated, an essential catalytic residue. In addition, the pH dependency data are interpreted to indicate that, contrary to earlier suggestions, the negatively charged side chain of Asp158 does not significantly stabilize the active-site thiolate-imidazolium ion pair. However, its presence does influence the pK_a's associated with ion-pair formation in a manner compatible with electrostatic considerations.

Since papain was first described in 1879 (Wurtz & Bouchut, 1879), a large database of information on the enzyme has been gathered. In particular, evidence has accumulated for the formation of an ion pair at neutral pH between residues Cys25 and His159, and it has been proposed that this is the active form of the enzyme [e.g., Polgar (1974), Sluyterman and

Wijdenes (1976), Halasz and Polgar (1977), Lewis et al. (1981), and Migliorini and Creighton (1986)]. For an in-depth review of cysteine proteases in general and papain in particular, see Brocklehurst et al. (1987). Mechanistically and kinetically, papain has been extensively characterized, and the structure of the native enzyme has been determined at 1.65-Å resolution (Kamphuis et al., 1984). In addition, the structures of several papain-inhibitor complexes are also available (Drenth et al., 1976; Varughese et al., 1989). These facts together with the recent construction of a synthetic gene coding for the papain precursor (Vernet et al., 1989) and its expression in a bacu-

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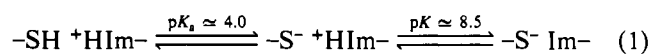
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lovirus-insect cell system (Vernet et al., 1990) make papain an ideal candidate for functional studies by site-directed mutagenesis.

The general catalytic mechanism of papain is well understood, but there has been some controversy concerning the mechanistic role of several active-site residues. Two such residues are Gln19 and Asp158. The former may or may not assist catalysis through the binding of the oxyanion of the tetrahedral intermediate, and the latter has had several mechanistic roles suggested. In particular, in earlier studies it was thought that Asp158, and not His159, was the residue acting as a general base in catalysis (Bender & Brubacher, 1966; Kirsch & Ingelström, 1966; Williams & Whitaker, 1968; Drenth et al., 1971; Löffler & Schneider, 1974). This idea has since been rejected and His159 is now widely considered to be the general base catalyst. A conformational change in which a His159-Asn175 hydrogen bond is broken to form a new conformational state with the negatively charged side chain of Asp158 interacting with the protonated imidazole of His159 has been proposed on the basis of cryoenzymological work (Angelides & Fink, 1978, 1979). However, further studies have discounted this possibility (Kamphuis et al., 1985; MacKenzie et al., 1985). Studies on substituted benzoyl-papains were also interpreted as evidence for the involvement of Asp158 in the deacylation process (Zannis & Kirsch, 1978).

In addition to the ambiguous conclusions reached from experimentation concerning Asp158 and its possible catalytic role, several theoretical studies have also suggested a role for this residue (Lavery et al., 1983; Dijkman et al., 1989; Rullman et al., 1989). In particular, an "experiment in computational site-directed mutagenesis" suggests that Asp158 is an active-site residue essential for catalysis to occur (Dijkman et al., 1989). From this and other theoretical studies it has been suggested that the ionized Asp158 side chain contributes significantly to the stabilization of the putative active-site thiolate-imidazolium ion pair formed between Cys25 and His159. Using calculations that disregard the contribution of solvent, Rullman et al. (1989) calculate that Asp158 substantially stabilizes the active-site zwitterion. However, they recognize that due to its location at the surface of the protein the electric field generated by the side chain of Asp158 is likely to be damped by solvent. In the parallel theoretical study, Dijkman et al. (1989) conclude that the effect of the protein matrix on the stabilization energy of the active site is dominated by the contribution of the negative charge of Asp158. It is suggested that this contribution is responsible for more than half of the effect of the entire protein on the active-site stability and in particular that Asp158 stabilizes the Cys-His zwitterion. In contrast to its effect on stability, Dijkman et al. (1989) conclude that Asp158 has a much smaller influence on the barrier to proton transfer within the zwitterion.

Additional experimental evidence has also been interpreted to support the view that the side chain of Asp158 has a direct influence on the catalytic activity of the enzyme. This evidence derives from studies on the pH dependence of k_{cat}/K_M for the papain-catalyzed hydrolysis of a variety of substrates. For example, with specific substrates this dependency was originally considered to be bell-shaped with pK_a 's of approximately 4.0 and 8.5. The acid limb of the profile was thought to represent the formation of the thiolate-imidazolium (Cys25-His159) ion pair and the alkaline limb the deprotonation of the ion pair, i.e.



However, more recently evidence has accumulated that an

additional acidic pK_a is involved; i.e., the acid limb of the pH profile is defined by two ionization steps, both with pK_a 's of approximately 4. These ionizations are positively cooperative, and it has been suggested that the additional pK_a is associated with the side-chain carboxylate of Asp158 (Bendall & Lowe, 1976; Lewis et al., 1978; Brocklehurst et al., 1983, 1984; Salih et al., 1987; Kowlessur et al., 1989).

It is difficult to reconcile the continued insistence on a role for Asp158 in catalysis when one considers that this residue is not conserved in all cysteine proteases. For example, cathepsin B has a glycine and cathepsin H an asparagine at the equivalent position in their sequences. Until the advent of site-directed mutagenesis no tool was available to obtain direct experimental evidence for the role of Asp158. However, now, through the availability of an expression system for papain (Vernet et al., 1990) coupled with site-directed mutagenesis, such experiments are possible. This report details a study in which the Asp158 residue of papain is replaced by an asparagine residue.

MATERIALS AND METHODS

Papain (2× crystallized suspension in sodium acetate) and DTNB¹ were obtained from Sigma Chemical Co. Papain was further purified on a mercurial agarose column by a method described previously (Sluyterman & Wijdenes, 1970). The enzyme is activated by addition of β -mercaptoethanol followed by gel filtration on Sephadex G-15. The substrate CBZ-Phe-Arg-MCA and the inhibitor E-64 were purchased from IAF Biochem International Inc., Laval, Québec. Thio-propyl-Sepharose was obtained from Pharmacia.

Site-Directed Mutagenesis. A 1048-bp gene coding for the precursor of papain was assembled from chemically synthesized oligodeoxyribonucleotides (Vernet et al., 1989). The gene was cloned into IpDC125, a version of the baculovirus expression vector pAc373 (Smith et al., 1985) containing the ϕ 1 phage origin of replication (Vialard et al., 1990). The Asp158Asn² mutation was made by in vitro site-directed mutagenesis (Zoller & Smith, 1982; Kunkel, 1985), creating the plasmid IpDC148. The oligonucleotide used for mutagenesis, 5'-TAACAAGGTCAATCATGCTGTTGC-3', also destroys a *SalI* restriction site, a feature used for facile diagnosis of the mutation. The entire papain precursor gene was sequenced before and after mutagenesis to ensure that no alterations other than the desired mutation were introduced into the gene. Construction of the recombinant baculovirus was by cell-mediated homologous recombination between plasmid IpDC148 and *Autographa californica* nuclear polyhedrosis virus genome (Vialard et al., 1990). Recombinant virus stocks were checked by DNA sequencing of the virus genome in the region of the papain gene around position Asp158 after amplification of a DNA fragment by the polymerase chain reaction with oligonucleotide primers flanking the mutation site (Saiki et al., 1988). *Spodoptera frugiperda* Sf9 cells were grown in spinner flasks and infected as described (Vialard et al., 1990). The infection with the recombinant virus leads to the secretion of the papain precursor (Vernet et al., 1990).

Purification of Recombinant Intracellular Papain. Infected cells in suspension culture (500 mL) were centrifuged at 1800g

¹ Abbreviations: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); CBZ-Phe-Arg-MCA, carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumarinyl-7-amide hydrochloride; E-64, 1-[[[L-trans-epoxysuccinyl]-L-leucyl]amino]-4-guanidino)butane; MCA, 7-amino-4-methylcoumarin.

² The amino acid numbering of the mature enzyme starting at Ile1 is used.

for 10 min. The pellet was extracted in 50 mL of 0.1 M sodium acetate buffer, pH 4.0, containing 20 mM cysteine, and the extract was incubated at 60 °C for 45 min. The extract was then centrifuged at 3000g for 5 min. The proteins in the supernatant were precipitated by the addition of solid ammonium sulfate to 80% salt saturation and were collected by centrifugation. The resulting ammonium sulfate pellet was suspended in 10 mL of 0.1 M acetate buffer, pH 4.0, containing 1 mM EDTA (buffer A) and was subjected to covalent chromatography on a thiopropyl-Sepharose column (15 × 5 mm i.d.) that had previously been equilibrated with the same buffer. The column was washed with 10 bed volumes of buffer A followed by 10 bed volumes of 0.1 M Tris-HCl buffer, pH 8.0, containing 1 mM EDTA (Buffer B). Papain was eluted with 0.1 M Tris-HCl buffer, pH 6.0, containing 20 mM cysteine, 1 mM DTT, and 1 mM EDTA.

Purification of Recombinant Secreted Papain. Typically 500 mL of medium was concentrated 10-fold by ultrafiltration using an Amicon ultrafiltration cell fitted with a PM10 membrane. Cysteine was added to the concentrated medium to a final concentration of 20 mM, and the mixture was adjusted to pH 4.0 by the addition of 0.1 M acetate buffer. The mixture was incubated at 60 °C for 45 min to activate processing of the precursor to mature papain, and the extract was then centrifuged at 3000g for 5 min. The supernatant was then treated with solid ammonium sulfate and the recombinant papain was purified as described above.

Enzyme Assay. For the standard assay used during purification, the reaction mixture consisted of 0.1 M phosphate buffer, pH 6.0, containing 2 mM cysteine, 1 mM EDTA, 0.08% Brij 35, and 5 μM CBZ-Phe-Arg-MCA. The reaction was initiated by the addition of enzyme, and the fluorescence of the MCA product was monitored in a Cary 2200 spectrophotometer equipped with a fluorescence accessory. Total fluorescence was measured by using an excitation wavelength of 380 nm and a Type GG455 cutoff filter in the emission beam.

Kinetic Measurements. All kinetic experiments were performed at 25 °C. Glassware used to keep enzyme solutions was silanized in order to minimize loss of enzyme at very low protein concentrations by adsorption to glassware.

The concentration of active commercial papain was determined with DTNB as described by Ellman (1959). Due to the low concentration of enzyme and to the presence of cysteine in the stock solutions, the concentration of mutant enzyme was determined by active-site titration with the potent inhibitor E-64 (Barrett & Kirschke, 1981). The mutant enzyme and E-64 were incubated at 25 °C to ensure complete reaction (15–60 min), after which an aliquot was withdrawn and assayed for remaining activity with CBZ-Phe-Arg-MCA as substrate. For kinetic measurements the standard assay conditions consisted of 50 mM phosphate buffer, 0.2 M NaCl, 5 mM EDTA, and 20% CH₃CN, pH 6.5.

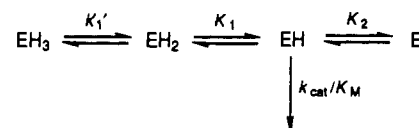
Kinetic measurements were performed by monitoring the fluorescence of MCA [λ (excitation) = 380 nm, λ (emission) = 440 nm] released during hydrolysis of the substrate. Measurements were done on a Perkin-Elmer LS-5B fluorometer interfaced to an Olivetti computer via an ADALAB-PC A/D converter (Interactive Microwave Inc.). LABTECH NOTEBOOK software (Laboratory Technologies Corp.) was used for data acquisition and storage. The response of the fluorometer was calibrated by using standard solutions of MCA prepared in the medium used for enzymatic reactions. Initial rates were evaluated by linear least-squares fit on the initial portion of emission curves. For pH-activity profiles, buffers

were used that gave the following final concentrations: 50 mM sodium citrate (pH 3.0–5.8), 50 mM sodium phosphate (pH 5.8–7.9), and 50 mM sodium borate (pH 8.0–10.0). Ionic strength was adjusted with NaCl. All reaction mixtures contained 5 mM EDTA (except solutions containing sodium citrate, where the EDTA concentration was 1 mM) and 20% CH₃CN. The value and stability of pH for each reaction was checked immediately after measurement of the initial rate.

The kinetic parameters for the hydrolysis of CBZ-Phe-Arg-MCA by commercial papain, recombinant wild type, and the Asp158Asn mutant were determined at near-optimum pH (6.5–6.7) by measuring initial rates at substrate concentrations ranging from 0.004 to 1.00 mM. Since the pK_a 's of the acid and alkaline limbs of the pH-activity profiles are separated by at least 4 pK_a units, the parameters obtained at optimum pH reflect the pH-independent values for the fully active form of the enzyme. The parameters were obtained by linear regression of the data to plots of s/v vs s (Hanes plots). Experiments in our laboratory indicate that, under the conditions used, the error on initial rate measurements is proportional to the magnitude of the initial rate. For relative errors a linear regression of this type is statistically correct (Cornish-Bowden, 1976).

In order to obtain pH profiles for k_{cat}/K_M , experiments were conducted at a substrate concentration such that $s \ll K_M$. Under these conditions the initial rate measured, v_i , is approximated by $v_i = e_0 s (k_{cat}/K_M)_{obs}$ where e_0 is the enzyme concentration used. With this relationship, values of $(k_{cat}/K_M)_{obs}$ were obtained from v_i measurements at several values of pH. For analysis of the pH-activity data, two models are used to describe the bell-shaped profiles that are obtained. For high-resolution data a four-protonation-state model is used:

model 1

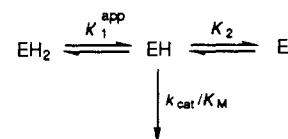


In this model only the species EH is active, and the pH-activity profile is defined by the equation

$$(k_{cat}/K_M)_{obs} = \frac{k_{cat}/K_M}{\frac{[H^+]^2}{K_1'K_1} + \frac{[H^+]}{K_1} + 1 + \frac{K_2}{[H^+]}} \quad (2)$$

where k_{cat}/K_M is the specificity constant for the fully active form. For low-resolution data model 2, a simplified form of model 1, is used:

model 2



For this model

$$(k_{cat}/K_M)_{obs} = \frac{k_{cat}/K_M}{\frac{[H^+]}{K_1^{app}} + 1 + \frac{K_2}{[H^+]}} \quad (3)$$

This simplified model is used primarily for qualitative comparisons of profile shapes, and model 1 is used for quantitative comparisons of pK_a shifts. Additionally, in order to simplify the fitting of data to eq 2, an initial fit is made by using the full pH range and eq 3 (model 2). This is then followed by the fitting of the data in the pH 3.0–6.5 range to eq 2. In this

Table I: Purification of Recombinant Papain^a

| step | total protein (mg) | | sp act. (s ⁻¹) | | purification (x-fold) | | recovered (%) | |
|----------------------|--------------------|------|----------------------------|-------|-----------------------|------|---------------|-----|
| | S | IC | S | IC | S | IC | S | IC |
| crude | 25 | 30 | 0.038 | 0.039 | | | 100 | 100 |
| ammonium sulfate | 22 | 28 | 0.039 | 0.043 | 1.02 | 1.1 | 90 | 85 |
| thiopropyl-Sepharose | 0.1 | 0.09 | 43.5 | 39 | 1160 | 1000 | 51 | 59 |

^aS, secreted recombinant papain; IC, intracellular recombinant papain. Values are based on 500-mL cultures.

second fit, K_2 is treated as a constant with a value equal to that obtained from the fit to model 2 (eq 3). To evaluate with as much precision as possible the two acid pK_a 's in model 1 (pK_1' and pK_1), many pH profiles were obtained in the low pH region ($pH \leq 6.5$). For reactions at very low pH ($pH < 3.5$), enzyme inactivation in the buffer was observed. This problem was greatly reduced by increasing the enzyme concentration and minimizing the mixing time. However, reliable data were not always obtainable below pH 3.2. Consequently these data points were discarded.

Theory. To account theoretically for the shift in the pK_a values due to the mutation of Asp158 to Asn in papain, we have used the general assumption that electrostatic interactions are mainly responsible for the observed effects. Following Tanford and Roxby (1972), it was assumed that the effect of the charge distribution on the pK_a of a particular group i (pK_i) is determined by the interaction of all the other charges of the molecule with the charged form of the i th group. The pK_i can then be related to its intrinsic pK_i^{int} by the relation

$$pK_i = pK_i^{int} + \delta pK_i \quad (4)$$

where

$$\delta pK_i = -W_i/2.303z_i k_B T \quad (5)$$

where W_i is the work done to place a charge, $z_i e$ (e is the proton charge), in the i th site for a given constellation of charges for the rest of the protein, k_B is the Boltzmann constant, and T is the temperature. Mutation of a residue with a ionizable group (k th ionizable site), in the neighborhood of the i th dissociable site, to a noncharged residue will alter the pK_a of the group at the i th site. This pK_a shift can be estimated by using the relation

$$\Delta pK_i = \Delta W_i/2.303z_i k_B T \quad (6)$$

with

$$\Delta W_i = W_i - W_i' \quad (7)$$

where W_i' represents the work of charging the i th site in the mutated molecule.

If it is assumed that the mutation does not considerably influence other ionizable groups except that at the i th site, i.e., if there is no other site in the neighborhood of the residue being mutated that dissociates with a similar dissociation constant, then the shift in pK_a of the i th site can be related to the difference between the work, $W_{i,k}$, of charging the i th site in the presence of a charge in the k th site and the work, $W_{i,k}'$, of charging it in the absence of charge in the k th site. This can be summarized by the equation:

$$\Delta W_i \cong W_{i,k} - W_{i,k}' \quad (8)$$

$W_{i,k}$ (or $W_{i,k}'$) can be evaluated as the work involved in gradually increasing the charge on the i th site by small increments, de , which is given by the integral

$$W_{i,k} = \int_0^{z_i e} \psi_i de \quad (9)$$

where $z_i e$ is the full charge of the i th site and ψ_i is the potential at that site.

Calculation of the Electrostatic Potential. The evaluation of the electrostatic potential is accomplished by using the macromolecular electrostatic package DelPhi (Klapper et al., 1986; Sharp et al., 1987; Gilson et al., 1987; Gilson & Honig, 1988). This package includes a finite-difference algorithm to solve the linearized Poisson-Boltzmann equation. The solvent and the protein molecule are treated as homogeneous dielectric media. The latter is approximated by a dielectric cavity whose shape was computed from the crystal structure of the protein (Kamphuis et al., 1985); a set of charges are immersed within this cavity, assigning them to points that coincide with the location of selected atoms that can either be ionized or partially charged. The solvent is represented as a medium with dielectric constant of 78.3 containing counterions that screen the fixed charges according to the Debye-Hückel model. The numerical solution of the Poisson-Boltzmann equation was carried out by using a cubic grid $65 \times 65 \times 65$ points. The feature of *focusing* provided within the DelPhi program was used. An ionic strength of 0.35 M was used in these calculations.

The computation of the integral given by eq 9 has to be done numerically. However, we carried out a series of tests that showed a linear relationship between the potential at a given site and the amount of charge assigned to it. By using a linear dependence of the potential with the charge,³ the work, $W_{i,k}$, can be expressed as

$$W_{i,k} = (\psi_i^{final} + \psi_i^{init})z_i/2 \quad (10)$$

where ψ_i^{init} and ψ_i^{final} are the initial and final values of the potential at the i th site.

RESULTS

Purification. Expression of the gene for prepropapain in insect cells results in partial secretion of the enzyme as glycosylated propapain, and the remaining enzyme is retained as a mixture of glycosylated propapain and prepropapain by the cells (Vernet et al., 1990). It was considered important, therefore, to characterize both the secreted and the intracellular protein and compare their enzymatic properties with those of natural papain. Recombinant papain was purified by covalent chromatography on a thiopropyl-Sepharose column. This resulted in a 1160-fold increase in specific activity of the enzyme preparation as compared with the culture media for the secreted protein. There was also a 1000-fold increase in specific activity of the intracellular protein when compared with the initial supernatant obtained from infected cells (Table I).

The purity and primary structure of the purified recombinant protein were verified by amino acid and N-terminal sequence analyses (Vernet et al., 1990; data not shown). The results were compared with those obtained by using commercially available papain. The amino acid composition results indicate that the recombinant protein is similar to commercial papain. However, from N-terminal sequences of the intra-

³ A linear dependence of the potential with charge is obtained for a spherical cavity, under the assumption that the charge is uniformly distributed on its surface.

Table II: Kinetic Parameters for CBZ-Phe-Arg-MCA Hydrolysis^a

| | k_{cat} (s ⁻¹) | K_M (mM) | k_{cat}/K_M (M ⁻¹ ·s ⁻¹) | $\text{p}K_1^{\text{app}}$ | $\text{p}K_2$ |
|--------------------------------|-------------------------------------|-----------------|--|----------------------------|-----------------|
| commercial papain ^b | 52 ± 6 (3) ^c | 0.42 ± 0.07 (3) | (1.2 ± 0.2) × 10 ⁵ (10) | 4.60 ± 0.07 (9) | 8.54 ± 0.10 (4) |
| recombinant wild-type | 51 ± 17 (4) | 0.40 ± 0.09 (4) | (1.2 ± 0.2) × 10 ⁵ (4) | 4.62 ± 0.07 (4) | 8.52 ± 0.04 (4) |
| Asp158Asn | 34 ± 6 (4) | 1.8 ± 0.5 (4) | (2.0 ± 0.2) × 10 ⁴ (4) | 4.34 ± 0.03 (6) | 8.18 ± 0.04 (5) |

^a Values of k_{cat} , K_M , and k_{cat}/K_M are from studies at optimum pH using various substrate concentrations, while $\text{p}K_1^{\text{app}}$ and $\text{p}K_2$ values were obtained from pH-activity profiles. ^b k_{cat}/K_M for papain also includes values determined from pH-activity profiles. ^c Value in parentheses indicates number of duplicate experiments.

cellular and the secreted recombinant papains it was determined that the *in vitro* site of processing of the proenzyme results in a ragged N-terminus. In addition to processing at the correct site, processing also occurs at sites 3 and 5 amino acids upstream into the pro region of the precursor protein (Vernet et al., 1990).

Kinetic Characterization at Optimum pH. Within experimental error the kinetic parameters obtained for the recombinant wild-type papain were independent of enzyme source, i.e., intracellular or extracellular, and values of k_{cat} and K_M for this enzyme are 51 s⁻¹ and 0.40 mM, respectively, with a k_{cat}/K_M of 1.2 × 10⁵ M⁻¹·s⁻¹. In addition, these results agree with the parameters obtained for commercial papain under the same conditions (Table II). For the Asp158Asn mutant an approximately 6-fold decrease in the specificity constant k_{cat}/K_M was observed (Table II). This decrease is accounted for mainly by an increase in K_M , while the k_{cat} value for the mutant enzyme shows a relatively small decrease.

pH-Activity Profiles. The pH dependence of $(k_{\text{cat}}/K_M)_{\text{obs}}$ reflects ionizations of the free enzyme and free substrate that are either directly involved in catalysis or responsible for maintaining the active conformation of the enzyme or substrate (Fersht, 1985). The only ionizable group on the substrate, arginine, remains protonated in the pH range used for our measurements (pH 3–10). Therefore, the pH dependence of $(k_{\text{cat}}/K_M)_{\text{obs}}$ will solely reflect the ionization of groups on the free enzyme. The bell-shaped profile can be explained, to a first approximation, by model 2, where the enzyme exists in three protonic forms (EH₂, EH, and E), only one of them being active. Nonlinear regression of the experimental data to eq 3 allows determination of the $\text{p}K_a$ values characterizing model 2 (Table II). The parameters for commercial papain and the recombinant wild-type papain are in very good agreement. For the Asp158Asn mutant however, a significant downward shift in the $\text{p}K_a$'s is evident (Table II) and is clearly shown in Figure 1.

Presence of an Additional Residue Modulating Activity at Low pH. On examination of Figure 2, where the low pH regions of the pH-activity profiles for both commercial papain and the Asp158Asn mutant have been plotted, it can be seen that the acid portion of the pH-activity profile is slightly steeper than expected for a dependence on a single ionizable group. This indicates the existence of a second group affecting the activity of the enzyme at low pH. The minimum model describing such a system, model 1, takes into account four protonic states of the enzyme (EH₃, EH₂, EH, and E) and considers that only one form is active (EH). The pH-activity profile for this model is described by eq 2.

Agreement between the calculated curve and experimental data for papain is much better for a model considering two ionizations rather than one in the acid pH range as shown in Figure 2a. The $\text{p}K_a$'s of the residues involved are 4.44 and 3.9. A similar experiment with the Asp158Asn mutant clearly demonstrates that once again the decrease in activity at low pH is best described by a two-ionization process (Figure 2b). The values of $\text{p}K_1$ and $\text{p}K_1'$ obtained for the mutant are both lower than the ones for commercial papain.

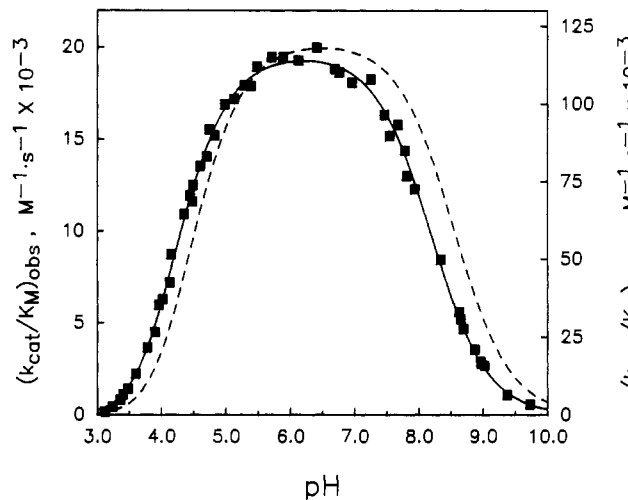


FIGURE 1: pH dependence of $(k_{\text{cat}}/K_M)_{\text{obs}}$ for the Asp158Asn mutant. At each pH, $(k_{\text{cat}}/K_M)_{\text{obs}}$ was determined by measuring the initial rate of CBZ-Phe-Arg-MCA hydrolysis and dividing by enzyme and substrate concentrations. The substrate concentration was kept constant at 0.06 mM. The solid line is the best fit to model 1 (see text). The dashed line is the best fit for data (not shown) obtained by using commercial papain.

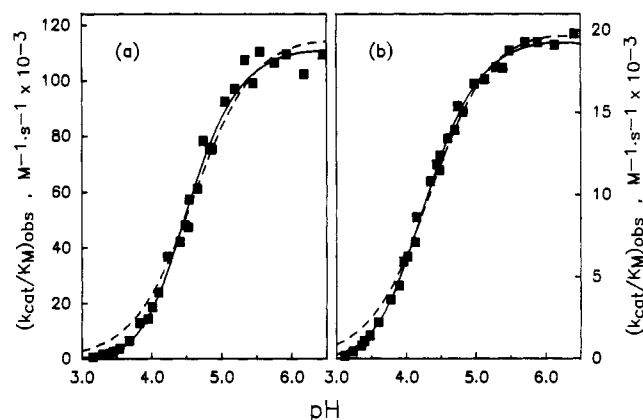


FIGURE 2: pH dependence of $(k_{\text{cat}}/K_M)_{\text{obs}}$ for (a) commercial papain and (b) Asp158Asn in the low pH region. In each case, the dashed line is the best fit to model 2. The solid line represents the best fit to model 1, with $\text{p}K_1 = 4.44 \pm 0.03$ and $\text{p}K_1' = 3.9 \pm 0.2$ for commercial papain (averages of five determinations) and $\text{p}K_1 = 4.22 \pm 0.04$ and $\text{p}K_1' = 3.6 \pm 0.1$ for the Asp158Asn mutant (averages of four determinations).

Calculated $\Delta\text{p}K_a$'s. Different values of the dielectric constant for the protein cavity have been assumed in order to estimate the observed $\text{p}K_a$ shifts. A low dielectric constant of 2 was not found to be compatible with the experimental results, since the predicted $\Delta\text{p}K_a$'s were 1 order of magnitude larger than the observed ones.

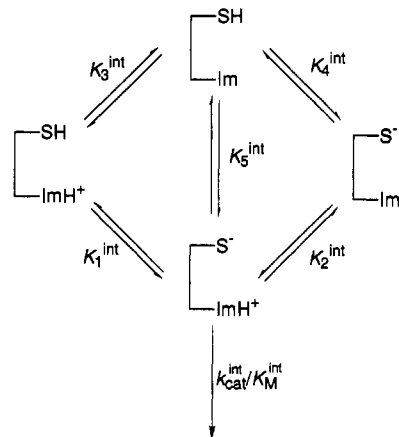
Table III presents a summary of the calculations, where a dielectric constant of 50 was used. These calculations were carried out for the four species in Scheme I. This scheme includes both the uncharged and zwitterion forms of the active-site residues. Calculations involving the wild-type papain were carried out under the assumption that the charge in

Table III: Electrostatic Potential Values at Selected Atoms in Wild-Type and Asp158Asn Papains and Electrostatic Free Energies Associated with the Charge Distribution

| molecule | state ^a | free energy ^b (kcal/mol) | atom ^c | charge (e) | potential ($k_B T/\epsilon$) |
|-----------|-------------------------------------|-------------------------------------|-------------------|------------|--------------------------------|
| wild type | -SH, -ImH ⁺ | 6.46 | S _γ | 0.0 | 1.7 |
| | | | N _{δ1} | 1.0 | 14.2 |
| | | | O _{δ1} | -0.5 | -8.7 |
| | | | O _{δ2} | -0.5 | -6.0 |
| | -S ⁻ , -ImH ⁺ | 9.11 | S _γ | -1.0 | -10.5 |
| | | | N _{δ1} | 1.0 | 11.9 |
| | | | O _{δ1} | -0.5 | -9.4 |
| | | | O _{δ2} | -0.5 | -6.5 |
| | -SH, -Im | 2.49 | S _γ | 0.0 | -0.6 |
| | | | N _{δ1} | 0.0 | -1.0 |
| | | | O _{δ1} | -0.5 | -9.9 |
| | | | O _{δ2} | -0.5 | -6.7 |
| | -S ⁻ , -Im | 6.50 | S _γ | -1.0 | -12.8 |
| | | | N _{δ1} | 0.0 | -3.3 |
| | | | O _{δ1} | -0.5 | -10.6 |
| | | | O _{δ2} | -0.5 | -7.2 |
| Asp158Asn | -SH, -ImH ⁺ | 4.55 | S _γ | 0.0 | 2.3 |
| | | | N _{δ1} | 1.0 | 15.2 |
| | -S ⁻ , -ImH ⁺ | 6.86 | S _γ | -1.0 | -10.0 |
| | | | N _{δ1} | 1.0 | 12.9 |
| | -SH, -Im | 0.00 | S _γ | 0.0 | 0.0 |
| | | | N _{δ1} | 0.0 | 0.0 |
| | -S ⁻ , -Im | 3.67 | S _γ | -1.0 | -12.2 |
| | | | N _{δ1} | 0.0 | -2.3 |

^aSee Scheme I. ^bA temperature of 300 K was assumed for these calculations. ^cS_γ refers to the Cys25 S_γ atom, N_{δ1} refers to the His159 N_{δ1} atom, and O_{δ1} and O_{δ2} refer to Asp158 O_{δ1} and O_{δ2} atoms, respectively.

Scheme I



Asp158 was equally distributed between the two oxygens of the carboxylic group. Under the label "potential", the values of the electrostatic potential (ψ_i) evaluated at the center of the corresponding atoms are displayed. The electrostatic free energy of each state (or charge distribution), displayed in the third column of Table III, was evaluated by using the relation

$$F = \sum_i z_i \epsilon \psi_i \quad (11)$$

The data contained in Table III were used in eq 10 to evaluate the work of charging the appropriate sites. Then, by means of eqs 8 and 6 we were able to derive the values of -0.25 and -0.42 for ΔpK_1^{int} and ΔpK_2^{int} , respectively. These values compare favorably with the measured ΔpK_a 's of -0.22, -0.3, and -0.34 for pK_1 , pK_1' , and pK_2 , respectively (Table II and Figure 2). The assignment of pK_1^{int} to either pK_1 or pK_1' is discussed below.

The calculations also indicate that the mutation of Asp158 to Asn shifts the equilibrium between -SH, -Im and -S⁻,

-ImH⁺ (Scheme I, K_3^{int}) toward the nonionized form by only 0.24 kcal/mol, indicating that the negative charge on Asp158 plays a minor role in the stabilization of the thiolate-imidazolium active-site ion pair.

DISCUSSION

With respect to the substrate used for this study, the activity and pH dependence of the recombinant wild-type papain is not discernably affected by either its source, intra- or extracellular, or the additional N-terminal extensions, which are absent from the commercial enzyme. Thus we are confident that the enzyme produced by genetic engineering is similar to commercially available papain. So far we have not been able to detect any kinetic or enzymatic differences between enzymes from these different sources. However, the Asp158Asn papain exhibits clear kinetic and pH dependence differences from the wild-type enzyme.

The replacement of the side chain of an aspartic acid by that of an asparagine within a protein, while producing a minimal change in van der Waals surface, eliminates entirely the potential for the side chain to ionize. This change is expected, therefore, to display a major influence on the electrostatic potential of the protein only in the pH range in which the acid side chain would be deprotonated. Also, due to the similar volume and shape of the Asp and Asn side chains, only minor steric influences on the protein structure induced by the mutation are expected. The difference in hydrogen-bonding capabilities between the Asp and Asn side chains could be expected to result in changes in side-chain orientation and possible localized conformational changes. Such was shown to be the case for an Asp102Asn trypsin (Sprang et al., 1987). In the wild-type trypsin both carboxylate oxygens of the Asp side chain participate as hydrogen-bond acceptors. One accepts hydrogen bonds from two main-chain amide groups, and the second accepts hydrogen bonds from both the N_{δ1} atom of His57 and the O_γ atom of Ser214. In the Asp102Asn trypsin, the carbonyl oxygen of the asparagine accepts hydrogen bonds from the same two main-chain amide groups, whereas the N_{δ2} atom of Asn102 donates hydrogen bonds to the O_γ of Ser214 and to the N_{δ1} atom of His57. In a comparison of the X-ray structures of the wild-type and Asp102Asn trypsins, the only significant structural change involves His57 and is linked to its protonation state; i.e., protonated histidine cannot act as a hydrogen-bond acceptor to the N_{δ2} of Asn102, and the steric constraint introduced is relieved through a His57 side-chain conformational change (Sprang et al., 1987).

In wild-type papain one side-chain carboxylate oxygen (O_{δ1}) of Asp158 forms hydrogen bonds with the main-chain NH groups of Ala136 and His159 (O...N distances 2.73 and 3.03 Å, respectively) (Baker & Drenth, 1987). The second carboxylate oxygen (O_{δ2}) according to the high-resolution structure of Kamphuis et al. (1984) is exposed to solvent and has a single hydrogen-bond partner, a tightly bound water molecule, W38 (O...O distance 2.6 Å). This water molecule in turn hydrogen-bonds to the main-chain carbonyl oxygen of residue 156 (O...O distance 2.75 Å). By replacing the second carboxylate oxygen of Asp158 with the N_{δ2} atom of Asn in the Asp158Asn papain, it is possible to accommodate the side-chain modification by reorienting the water molecule W38 such that it is now a hydrogen-bond acceptor. Therefore, this side-chain modification should not result in any significant change in the structure of the enzyme.

On substituting Asn for Asp at position 158 in the sequence of papain, the changes induced in the kinetic parameters, k_{cat}/K_M and k_{cat} , are approximately 6-fold and 2-fold, re-

spectively. These changes can be considered to be relatively minor in comparison with the 10^4 -fold decrease in k_{cat}/K_M observed when the same substitution is made at position 102 in trypsin (Craik et al., 1987). Clearly Asp102 in trypsin can be considered as an "essential" catalytic residue whereas Asp158 in papain cannot be considered as such. In addition, the presence of two acid pK_a 's (pK_1 and pK_1') in the pH- $(k_{\text{cat}}/K_M)_{\text{obs}}$ profile (Figure 2b) for Asp158Asn papain, although shifted to lower pH, conclusively shows that Asp158 is not responsible for one of these ionizations. This contradicts previous suggestions that the ionization of the Asp158 side-chain carboxyl group modulates the activity of papain and that its effect is evidenced by the influence of an acid pK_a , other than that assigned to the thiolate-imidazolium ion pair, on the pH- $(k_{\text{cat}}/K_M)_{\text{obs}}$ profiles (Bendall & Lowe, 1976; Lewis et al., 1978; Brocklehurst et al., 1983, 1984; Salih et al., 1987; Kowlessur et al., 1989). Unfortunately, from the data presented above, it is not possible to determine which of the two measured acid pK_a 's, either pK_1 or pK_1' , corresponds to pK_1^{int} in Scheme I. However, from the similar shifts in magnitude of the two pK_a 's in the Asp158Asn mutant we can conclude that the two groups responsible for the pK_a 's (the thiol of Cys25 and one other as yet unknown) are approximately at the same distance from the carboxylate group of Asp158. The identification of the source of this second acid pK_a is currently being sought through additional site-directed mutagenesis experiments.

From theoretical studies (Dijkman et al., 1989; Rullman et al., 1989) it was concluded that, through its electrostatic influence, the ionized side chain of Asp158 played a significant role in the stabilization of the active-site ion pair. However, the experimental results presented above show that removal of this side chain results in modest changes in the kinetic parameters, clearly indicating that this group plays only a minor role in defining the enzyme's activity. In particular, the 6-fold decrease in k_{cat}/K_M could be accounted for by changes in any or all of three processes, i.e., binding, catalysis, or stability of the ion pair. If in Scheme I K_3^{int} and K_4^{int} represent the ionization of the thiol and imidazole groups in the absence of factors that function to stabilize the ion pair, then from the relationships $K_5^{\text{int}} = K_1^{\text{int}}/K_3^{\text{int}}$ and $K_5^{\text{int}} = K_2^{\text{int}}/K_4^{\text{int}}$, any effect that increases the stability of the ion pair (K_5^{int} increases) will cause an increase in K_1^{int} and a decrease in K_2^{int} and therefore will lead to a broadening of the pH-activity profile. Inversely, factors that destabilize the ion pair will give rise to a narrowing of the pH profile. Experimentally, we observe that the value of $pK_2 - pK_1$ or $pK_2 - pK_1'$ decreases by only 0.12 or 0.04 pH unit, values that are within the experimental error for these measurements. Thus the overall shape and width of the pH profile remains essentially constant, suggesting that the stability of the ion pair is not greatly affected by the mutation. This conclusion is reinforced by the computational results presented in Table III. These results were obtained by the fitting of theoretical parameters to the experimental data obtained in this study. It can be shown that the experimental values of K_1 , K_2 , and k_{cat}/K_M are related to the intrinsic K_1^{int} , K_2^{int} , and $k_{\text{cat}}^{\text{int}}/K_M^{\text{int}}$ of Scheme I by the relations

$$K_1 = K_1^{\text{int}}/\alpha \quad (12)$$

$$K_2 = K_2^{\text{int}}\alpha \quad (13)$$

$$k_{\text{cat}}/K_M = (k_{\text{cat}}^{\text{int}}/K_M^{\text{int}})\alpha \quad (14)$$

where $\alpha = K_5^{\text{int}}/(K_5^{\text{int}} + 1)$. The value of K_5^{int} has been determined to be in the range of 8–12 (Lewis et al., 1976) for the wild-type enzyme, and to a first approximation, the experimental values will reflect the intrinsic values of the pa-

rameters in Scheme I. Therefore, the calculated values of K_1^{int} and K_2^{int} are expected to approximate the observed ones. The changes in free energy on going from $-\text{SH}$, $-\text{Im}$ to $-\text{S}^-$, $-\text{ImH}^+$ for the wild-type and the Asp158Asn enzymes are 6.62 and 6.86 kcal/mol, respectively. This corresponds to only a 0.24 kcal/mol stabilization of the thiolate-imidazolium ion pair relative to the neutral form ($-\text{SH}$, $-\text{Im}$) of the enzyme by the negative charge on the Asp158 side chain and could contribute partly to the difference in k_{cat}/K_M values. Moreover, these calculations based on theoretical considerations have shown that the observed shifts in pK_a 's are compatible with the assumption that electrostatic interactions make the major contribution to the observed effects.

The effect of the negative charge on the ionization involved in forming the ion pair can be readily explained qualitatively by considering its effect on the two ionization steps, K_1^{int} and K_2^{int} (Scheme I), individually. From the high-resolution crystal structure of papain (Kamphuis et al., 1984), the average distances between the two side-chain carboxylate oxygen atoms and the sulfur atom of Cys25 is 8.3 Å, and the corresponding distance for the oxygen atoms and the $N_{\delta 1}$ atom of His159 is 6.3 Å. At neutral pH the macroscopic charge on the ion pair is effectively zero, and on this basis, the negative charge on Asp158 can be expected to have little influence on the ion pair. However, since the $N_{\delta 1}$ of His159 is somewhat closer, some influence can be expected, although it is probably minor. Below pK_1^{int} the overall charge on the Cys-His pair is positive (Scheme I), leading to a highly favorable charge-charge interaction with the negative charge on Asp158, resulting in a stabilization of $-\text{SH}$, $-\text{ImH}^+$ relative to $-\text{S}^-$, $-\text{ImH}^+$. Thus, as observed (Figure 1, Table II) and calculated (Table III), the presence of Asp158 can be expected to raise pK_1^{int} . The pK_a of the side chain of Asp158 is not known but it is expected to be in the same range as pK_1^{int} . Therefore, to some extent the effect of the Asp158 side chain on pK_1^{int} will be attenuated by the fractional protonation of Asp158. At high pH the Cys-His pair is negatively charged and present as $-\text{S}^-$, $-\text{Im}$. This will give rise to an unfavorable interaction with the negatively charged Asp158 side chain. This interaction can be expected to raise pK_2^{int} , as observed experimentally. Therefore, it can be argued that whereas the Asp158 side chain does not influence significantly the stability of the active-site thiolate-imidazolium ion pair of papain, it does have an influence on the pK_a 's defining the formation of the ion pair. The magnitudes of the pK_a shifts observed (approximately 0.3 pH unit) are quite large given the ionic strength (0.35 M) at which the experiments were performed. A ΔpK_a of approximately 0.7 pH unit was predicted by Pickersgill (1988) for the shift in pK_a of the active-site cysteine residue in papain due to the ionization of the Asp158 side chain. However, in Pickersgill's prediction the ionic strength is considered to be zero. Russell et al. (1987) measured the ΔpK_a for the active-site His64 residue of subtilisin following the mutation of selected acid side chains on the enzyme. From an interpolation of their data for an acid residue, Asp99, that is 12–13 Å from His64, a ΔpK_a of approximately 0.15 is expected at an ionic strength of 0.35 M.

Thus we have shown that the recombinant enzyme produced in insect cells, even though it has a ragged N-terminus, is kinetically indistinguishable from commercial papain. In addition, we have shown that Asp158 in papain is not an essential catalytic residue, and contrary to earlier suggestion, when negatively charged it does not appear to significantly stabilize the active-site thiolate-imidazolium ion pair, although its presence does influence pK_a 's associated with its formation.

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