Accelerated Publications

Contribution of the Glutamine 19 Side Chain to Transition-State Stabilization in the Oxyanion Hole of Papain†

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ABSTRACT: The existence of an oxyanion hole in cysteine proteases able to stabilize a transition-state complex in a manner analogous to that found with serine proteases has been the object of controversy for many years. In papain, the side chain of Gln19 forms one of the hydrogen-bond donors in the putative oxyanion hole, and its contribution to transition-state stabilization has been evaluated by site-directed mutagenesis. Mutation of Gln19 to Ala caused a decrease in k_{cat}/K_M for hydrolysis of CBZ-Phe-Arg-MCA, which is 7700 M^{-1} s^{-1} in the mutant enzyme as compared to 464 000 M^{-1} s^{-1} in wild-type papain. With a Gln19Ser variant, the activity is even lower, with a k_{cat}/K_M value of 760 M^{-1} s^{-1}. The 60- and 600-fold decreases in k_{cat}/K_M correspond to changes in free energy of catalysis of 2.4 and 3.8 kcal/mol for Gln19Ala and Gln19Ser, respectively. In both cases, the decrease in activity is in large part attributable to a decrease in k_{cat}, while K_M values are only slightly affected. These results indicate that the oxyanion hole is operational in the papain-catalyzed hydrolysis of CBZ-Phe-Arg-MCA and constitute the first direct evidence of a mechanistic requirement for oxyanion stabilization in the transition state of reactions catalyzed by cysteine proteases. The equilibrium constants K_i for inhibition of the papain mutants by the aldehyde Ac-Phe-Gly-CHO have also been determined. Contrary to the results with the substrate, mutation at position 19 of papain has a very small effect on binding of the inhibitor. This important finding indicates that different types of inhibitors and substrates utilize the catalytic machinery of papain to differing degrees. Implications for the role of oxyanion hole interactions in cysteine proteases are discussed.

As suggested by Pauling in the 1940s, enzymes can cause appreciable rate accelerations by preferentially binding the transition-state complex formed during the reaction (Pauling, 1946, 1948). Serine and cysteine protease catalyzed hydrolysis involves a transition-state complex that is inherently unstable due to the development of a negative charge on the carbonyl oxygen of the substrate. In serine proteases, stabilization of such a transition state is achieved by an arrangement of dipoles in a cavity that has been termed the oxyanion hole (Henderson, 1970; Henderson et al., 1971; Robertus et al., 1972; Matthews et al., 1975; Poulos et al., 1976; Asbóth & Polgár, 1983, Bryan et al., 1986; Wells et al., 1986; Carter & Wells, 1990). However, the existence of such a cavity in cysteine proteases remains controversial (Wolthers et al., 1971; Asbóth & Polgár, 1983; Asbóth et al., 1985; Mackenzie et al., 1986). Examination of X-ray structures of papain–inhibitor complexes (Drenth et al., 1975, 1976) indicate that the Gln19 side chain and the Cys25 amide proton are positioned to interact with the oxyanion of a papain–substrate transition state. On the other hand, the ability of papain to hydrolyze thionoesters has been considered as evidence against the contribution of oxyanion hole interactions in the catalytic mechanism of papain

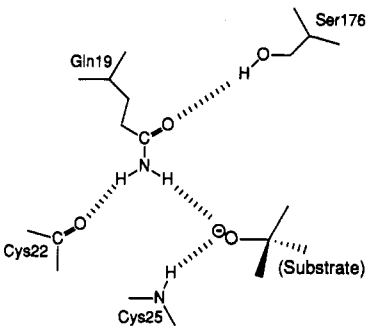

FIGURE 1: Schematic representation of the interactions involving the side chain of Gln19 and the amide proton of Cys25 in the oxyanion hole of papain.

(Asbóth & Polgár, 1983; Asbóth et al., 1985). Mackenzie et al. (1986) have also proposed that interactions between a tetrahedral intermediate and the oxyanion hole may not be possible in papain. Using ^{13}C NMR spectroscopy, they have shown that both *N*-acetyl-D- and *N*-acetyl-L-phenylalanylglycinals form thiohemiacetals with the same stereospecificity in the S_1 subsite of papain, and from computer modeling work, they have suggested that the hydroxyl function is not located in the oxyanion hole of papain.

Quantitative evaluation of transition-state stabilization in an enzyme-catalyzed reaction is an important aspect of enzymology. The advent of site-directed mutagenesis, however, has rendered such studies more accessible and allows structure–function correlations to be established. We have used this technique to investigate the contribution of interactions

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in the proposed oxyanion hole to rate enhancement with papain. Taking advantage of the fact that one of the hydrogen-bond donors, in the proposed oxyanion hole of papain, is provided by the side chain of Gln19 (Figure 1), two mutants of papain (Gln19Ala and Gln19Ser) have been expressed in the baculovirus–insect cell system (Vernet et al., 1990). The secreted precursors were converted in vitro to the mature enzymes and purified to homogeneity, and the kinetic parameters for hydrolysis of the CBZ-Phe-Arg-MCA[1] substrate and for inhibition by the aldehyde Ac-Phe-Gly-CHO have been determined. It will be shown that even though the enzyme–inhibitor complex and the enzyme–substrate transition state can be considered as analogues, they do not necessarily use the catalytic machinery of papain to the same degree.

Materials and Methods

Commercial papain was obtained from Sigma Chemical Co. and purified on a mercurial agarose column (Sluyterman & Wijdenes, 1970). The enzyme was activated and its concentration determined as described previously (Ménard et al., 1990). CBZ-Phe-Arg-MCA and E-64 were purchased from IAF Biochem International Inc., Laval, Québec. The peptidyl aldehyde Ac-Phe-Gly-CHO was a generous gift from Dr. M. Pozsgay (Institute for Biological Sciences, National Research Council of Canada).

Site-Directed Mutagenesis. The transfer plasmid IpDC127 (Vernet et al., 1990) containing a synthetic gene encoding the papain precursor (Vernet et al., 1989) was used as template for in vitro site-directed mutagenesis (Kunkel, 1985). Replacement of Gln19 by a Ser generated the transfer plasmid IpDC152 and was performed with the 5′-AGTTAAGA-ACTCAGGCAGCTGTGG-3′ oligodeoxyribonucleotide (oligo). The transfer plasmid IpDC153 containing an Ala at position 19 of papain was generated by using the 5′-AGTTAAGAACGCAGGCAGCTGTGG-3′ oligo. The former oligo created a *Dde*I restriction site upon mutagenesis whereas the latter destroyed a *Sty*I restriction site, two characteristics that were used to facilitate the identification of the mutated plasmid. The sequences of the entire papain precursor mutated genes were determined, and no alterations other than those designed were found. Construction of the recombinant baculoviruses, production of recombinant proteins in *Spodoptera frugiperda* Sf9 cells, and purification of the recombinant proteins were as described previously (Ménard et al., 1991b).

Kinetic Measurements. Kinetic experiments were performed at 25 °C. Hydrolysis of CBZ-Phe-Arg-MCA was followed by monitoring the fluorescence of MCA released during the reaction as described previously (Ménard et al., 1990). Concentration of active recombinant enzyme was determined by titration with E-64. Kinetic parameters were obtained by measuring the initial rate of hydrolysis at substrate concentrations ranging from 0.004 to 0.200 mM. Experimental conditions consisted of 50 mM phosphate buffer, 0.2 M NaCl, 5 mM EDTA, and 10% CH_3CN, pH 6.5. For pH–activity profiles, sodium citrate and sodium borate were also used as buffers. The dissociation constant K_i for inhibition of wild-type and mutant papain by Ac-Phe-Gly-CHO was determined by measuring the initial rate of substrate hydrolysis (CBZ-Phe-Arg-MCA) in the presence of varying concentrations of inhibitor and substrate. Under certain conditions, nonlinearity in the initial portion of the progress curve indicated the presence of a "slow-inhibiting" process and the rates were measured only after steady-state conditions were reached. The data was plotted as $1/v$ vs [inhibitor] (Dixon, 1953) for inhibition of wild-type papain by Ac-Phe-Gly-CHO. With the Gln19Ala and Gln19Ser mutants, the amount of inhibitor used in the assays was not in excess of the concentration of mutant enzyme, and the depletion of the inhibitor by the enzyme had to be taken into account when the data were analyzed (Knight, 1986). An estimated value of the apparent inhibition constant K_i^{app} was obtained by using a linear form of the equation describing tight-binding inhibition (Henderson, 1972). This value was then used as an initial estimate for the determination of K_i^{app} by nonlinear regression of the data to

$$v_i = \frac{k_{cat}[S]}{2(K_M + [S])}\{[(K_i^{app} + [I_0] + [E]_0)^2 - 4[E]_0[I]_0]^{1/2} - (K_i^{app} + [I]_0 - [E]_0)\} \quad (1)$$

where $K_i^{app} = K_i(1 + [S]/K_M)$ (Morrison, 1969). Values of K_i were not corrected for hydration of the aldehyde in aqueous solution.

[1] Abbreviations: CBZ-Phe-Arg-MCA, carbobenzoxy-L-phenylalanyl-(7-amino-4-methylcoumarinyl)-L-arginine; Ac-Phe-Gly-CHO, *N*-acetyl-L-phenylalanylglycinal; E-64, 1-[[(L-*trans*-epoxysuccinyl)-L-leucyl]amino]-4-guanidinobutane; MCA, 7-amino-4-methylcoumarin.

Table I: Kinetic Parameters for Hydrolysis of CBZ-Phe-Arg-MCA and Inhibition by Ac-Phe-Gly-CHO

enzyme	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (10^3 M^{-1} s^{-1})	$\Delta\Delta G^{\ddagger}$[a] (kcal/mol)	K_i[b] (nM)
wild type	41.6 ± 6.8	0.089 ± 0.006	464 ± 44		36 ± 5
Gln19Ala	2.4 ± 1.5	0.30 ± 0.10	7.7 ± 2.2	2.4	21 ± 6
Gln19Ser	0.23 ± 0.15	0.27 ± 0.13	0.76 ± 0.24	3.8	14 ± 9

[a] $\Delta\Delta G^{\ddagger}$ was obtained from the values of k_{cat}/K_M by using eq 1. [b] K_i represents the dissociation constant of the complex formed between enzyme and Ac-Phe-Gly-CHO, determined as described in the text.

Results

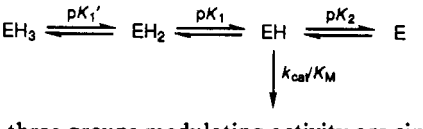
Kinetic Characterization of CBZ-Phe-Arg-MCA Hydrolysis. The results, reported in Table I, indicate that substitution of the amide side chain of Gln19 by a methyl group causes an appreciable 60-fold decrease in k_{cat}/K_M. The decrease is even more important for the Gln19Ser mutant, which is less active than wild-type papain by a factor of 610. Most of the effect is due to a decrease in k_{cat}, while the K_M value is only slightly affected by the mutations. The interaction energy of the oxyanion with the Gln19 residue in the transition state for the papain-catalyzed reaction can be evaluated by using

$$\Delta\Delta G^{\ddagger} = -RT \ln \left[\frac{(k_{cat}/K_M)_{mutant}}{(k_{cat}/K_M)_{wild\text{-}type}} \right] \quad (2)$$

where $\Delta\Delta G^{\ddagger}$ represents the change in free energy of transition-state stabilization on mutating the Gln19 residue. From the two mutations introduced at position 19, the side chain can be considered to contribute between 2.4 and 3.8 kcal/mol to transition-state stabilization.

The pH dependency of k_{cat}/K_M has also been determined for the Gln19Ala and Gln19Ser mutants (see Figure 2 for the Gln19Ala profile). The bell-shaped pH–activity profile can be described by the same model as used for papain (Ménard et al., 1990), where the activity is modulated by ionization of three groups on the enzyme:

model 1



The pK_a's of the three groups modulating activity are similar to those of papain but the pH–activity profiles are significantly

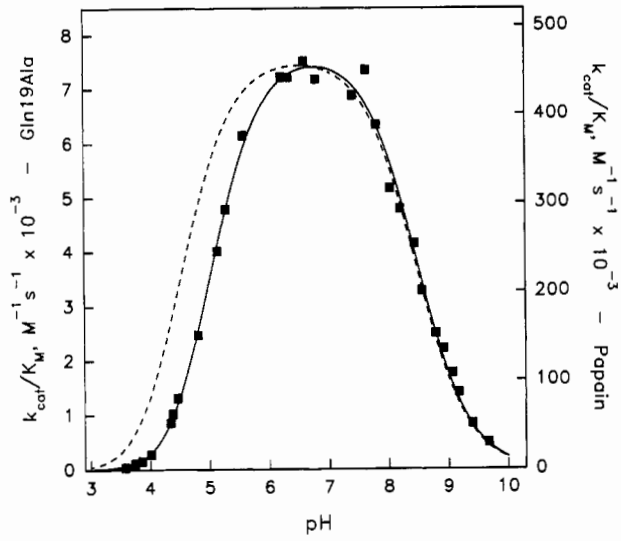


FIGURE 2: pH dependence of k_{cat}/K_M for hydrolysis of CBZ-Phe-Arg-MCA by the Gln19Ala mutant. Initial rates were determined at a substrate concentration much lower than the K_M (i.e., 0.04 mM) so that the k_{cat}/K_M value can be obtained by dividing the initial rate by enzyme and substrate concentrations (Ménard et al., 1990). The solid line represents the best fit of the data to model 1. The pK_a values obtained from the nonlinear regression analysis are pK_1' = 4.1 ± 0.2, pK_1 = 5.02 ± 0.04, and pK_2 = 8.51 ± 0.05. The dashed line represents the best fit for data (not shown) obtained with wild-type papain (pK_1' = 3.6 ± 0.3, pK_1 = 4.5 ± 0.3, and pK_2 = 8.45 ± 0.02). The corresponding experiment with the Gln19Ser mutant gave results similar to Gln19Ala papain; the pK_a's were pK_1 = 5.04 ± 0.03 and pK_2 = 8.33 ± 0.07. pK_1' could not be determined with Gln19Ser due to the lower activity of this enzyme.

narrower for the mutant enzymes and are shifted to higher pH values as compared to those of wild-type enzyme.

Inhibition by Ac-Phe-Gly-CHO. The dissociation constant K_i of the reversible complex formed between papain and Ac-Phe-Gly-CHO has been evaluated at 36 nM, a result in agreement with values determined by other groups (Westerick & Wolfenden, 1972; Mattis et al., 1977; Mackenzie et al., 1986). As shown in Table I, replacement of Gln19 by Ala or Ser has only a small effect on K_i, indicating that interaction of the inhibitor in the oxyanion hole of papain is either weak or absent.

Discussion

The large effect on k_{cat}/K_M for hydrolysis of CBZ-Phe-Arg-MCA observed with the papain mutants, Gln19Ala and Gln19Ser, is consistent with the existence of hydrogen bonding between the Gln19 side chain and the oxyanion of the substrate in the transition state. From the values of k_{cat}/K_M, this interaction can be evaluated to contribute between 2.4 and 3.8 kcal/mol toward transition-state stabilization. This constitutes the first direct evidence of a mechanistic requirement for oxyanion stabilization in a reaction catalyzed by a cysteine protease. By comparison, the stabilization energy of the transition state by the Asn155 side chain in subtilisin has been estimated from similar site-directed mutagenesis experiments to be 2.2–4.8 kcal/mol (Bryan et al., 1986; Wells et al., 1986; Rao et al., 1987; Carter & Wells, 1990). In particular, $\Delta\Delta G^{\ddagger}$ = 3.67 kcal/mol (Rao et al., 1987) for the substitution of Asn155 by an alanine in subtilisin, a value somewhat higher than that observed for Gln19Ala papain. However, it could be argued that a more accurate comparison would be between Gln19Ala papain and Asn155Gly subtilisin, since in both these mutants a CH_2CONH_2 fragment has been removed and replaced by a proton. The Asn155Gly substitution in subtilisin leads to a $\Delta\Delta G^{\ddagger}$ value of 3.2 kcal/mol (Carter & Wells, 1990), which is closer to the $\Delta\Delta G^{\ddagger}$ of 2.4 kcal/mol observed with Gln19Ala papain. These comparisons suggest that the extent of transition-state stabilization achieved through the oxyanion hole of papain, although highly significant, is somewhat smaller than that obtained through the analogous interaction in subtilisin.

The mutation of Gln19 to Ser has a larger effect on activity than the Gln19Ala mutation. This possibly reflects differing degrees of solvation of the oxyanion in these mutants, as a result of the different cavities created by the shortening of the Gln19 side chain. It is interesting that, with subtilisin, an Asn155 to threonine mutation has a much larger effect on activity than the corresponding alanine mutation (Wells et al., 1986; Rao et al., 1987), and this difference can be accounted for by theoretical calculations based on electrostatic considerations (Hwang & Warshel, 1987). Similarly, it is possible that the differing dipoles of the alanine and serine side chains could explain the difference in activity of the Gln19Ala and Gln19Ser mutants of papain.

The effect of the mutations at position 19 in papain is mainly on k_{cat} while K_M is only slightly affected. Similarly, mutations of Asn155 in the oxyanion hole of subtilisin were shown to have a major effect on k_{cat} while K_M remains virtually unchanged (Bryan et al., 1986; Wells et al., 1986; Rao et al., 1987; Carter & Wells, 1990). In the case of subtilisin, since acylation is rate limiting for the substrate used in the kinetic measurements, $K_M = K_s$, the dissociation constant for the ES complex. The absence of effect on K_M therefore indicates that the Asn155 side chain is not hydrogen bonded to the substrate carbonyl oxygen in the ground-state ES complex. The rate-limiting step for hydrolysis of CBZ-Phe-Arg-MCA by papain is not known, and interpretation of the variations in K_M is not as straightforward. However, the small changes in K_M observed for this enzyme do suggest that hydrogen bonding to the substrate carbonyl in the ground-state Michaelis complex is absent or weak.

Other possible consequences of these mutations on the activity of papain must also be considered before the effect is attributed solely to the removal of a Gln19-oxyanion interaction. Part of the observed decreases in activity for the Gln19Ala and Gln19Ser mutants could result from conformational changes brought by these mutations. The side chains of alanine and serine are relatively small in size and should not cause important modifications in conformation due to steric considerations. However, interactions of the side chain of Gln19 could be important for conformational integrity. The stability of the thiolate–imidazolium form of papain is very sensitive to conformational variations (Pickersgill, 1988; Ménard et al., 1991a), and the width of the pH–activity profile reflects the stability of this ion pair (Ménard et al., 1991b). Therefore, a conformational change that would have a large effect on activity would also be expected to have a major influence on the ion-pair stability. The observed narrowing for both Gln19Ala and Gln19Ser papain can be explained by a moderate to small change in ion-pair stability and does not suggest the presence of significant conformational changes. A particular interaction, the interdomain hydrogen bond between the carbonyl oxygen of the Gln19 side chain and the hydroxyl group of Ser176 ($O_{\epsilon 1}$–$O_\gamma H$ distance = 2.74 Å, see Figure 1), could possibly have a structural role important for catalysis by papain. However, mutation of residue 176 to an alanine was shown to cause only a minor decrease in activity (Ménard et al., 1991a), and therefore, the effect on activity of the mutations at position 19 of papain cannot be assigned to the removal of the Gln19–Ser176 hydrogen bond. The

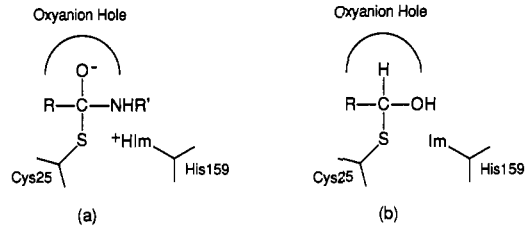


FIGURE 3: Postulated structures of (a) the tetrahedral intermediate in the papain-catalyzed hydrolysis of the peptidyl substrate CBZ-Phe-Arg-MCA and (b) the thiohemiacetal formed by reaction of papain with the inhibitor Ac-Phe-Gly-CHO.

Ser176Ala mutation caused a narrowing of the pH–activity profile comparable to that observed for the Gln19Ala and Gln19Ser mutants. This has been attributed to a destabilization of the thiolate–imidazolium ion pair of the Ser176Ala enzyme (Ménard et al., 1991a), and removal of the Gln19–Ser176 hydrogen bond by mutation of residue 19 is expected to have a similar effect. This by itself can explain the narrowing of pH–activity profiles for Gln19Ala and Gln19Ser and gives further argument against additional conformational changes detrimental to enzyme activity. The shifting of the pH–activity profiles to higher pH, observed for the Gln19 mutants as compared to wild-type or Ser176Ala papain, possibly reflects a change in the electrostatic potential of the active site upon removal of the dipole created by the Gln19 side chain (Ménard et al., 1990, 1991b).

The finding that oxyanion hole stabilization is operative in substrate hydrolysis by papain might seem in conflict with the observation that papain catalyzes the hydrolysis of specific thionoesters at a rate comparable with that of esters (Asbóth & Polgár, 1983; Asbóth et al., 1985; Storer & Carey, 1985). The complete loss of activity upon substitution of a sulfur atom in place of the carbonyl oxygen of the substrate with serine proteases has been attributed to perturbation of oxyanion hole interactions. From our results, it is clear that the ability of papain to hydrolyze thionoesters is not due to the absence of an oxyanion hole binding site and another explanation must be sought to account for the difference between serine and cysteine protease reactions with thionoesters. The interactions between the oxyanion and its binding site in serine proteases might be precluded or strongly affected by the substitution to a sulfur atom, while the papain–substrate complex can adjust to maintain these interactions as suggested previously by Storer and Carey (1985). A second explanation could lie in mechanistic differences of the two enzyme classes. Either suggestion is reasonable and could explain the ease by which thionoesters are hydrolyzed by papain. The work with thionoesters exemplifies well the previously encountered difficulties associated with providing definitive evidence for or against the role of an oxyanion hole in cysteine proteases. The fact that this study has positively identified a mechanistic role for this oxyanion hole is a clear demonstration of the power of protein engineering when applied to enzymological problems.

The effect of the mutations at position 19 on k_{cat}/K_M for hydrolysis of CBZ-Phe-Arg-MCA indicates that the negatively charged oxygen atom of the tetrahedral intermediate interacts with the Gln19 side chain in the oxyanion hole of papain as schematically depicted in Figure 3a. In this figure, the tetrahedral intermediate is shown for conversion of a peptidyl substrate to an acyl enzyme, with the protonated residue His159 in proximity to the nitrogen atom of the leaving group. The Gln19 mutations have been shown to have only a small effect on the binding of the peptidyl aldehyde Ac-Phe-Gly-CHO to papain. Aldehydes are very good reversible inhibitors of cysteine proteases, and their high potency has been attributed to the propensity of these compounds to complex with the enzyme to form structures that mimic the transition state of a protease-catalyzed reaction (Rich, 1986). Our results indicate that the inhibitor bound to papain does not interact with the Gln19 side chain as does the oxyanion in the transition-state of the CBZ-Phe-Arg-MCA hydrolysis reaction. This is in agreement with the suggestion of Mackenzie et al. (1986) that the hydroxyl group of the tetrahedral thiohemiacetal is not located in the oxyanion hole of papain, as shown in Figure 3b. However, this cannot be taken as evidence against the existence of an operative oxyanion hole for substrate hydrolysis by papain and cysteine proteases in general. In Figure 3b, the aldehyde forms a thiohemiacetal that does mimic the structure of a tetrahedral intermediate, with the hydroxyl group not ionized and the imidazole side chain of His159 deprotonated (Frankfater & Kuppy, 1981). Thiohemiacetal formation from reaction of the aldehyde with the thiolate–imidazolium ion pair of papain results in charge neutralization, whereas formation of the tetrahedral intermediate during substrate hydrolysis results in charge transfer from the sulfur atom of Cys25 to the carbonyl oxygen of the substrate. Interactions in the oxyanion hole are necessary to maintain the charge separation in the latter complex while they are not used with the neutral thiohemiacetal complex. This finding has tremendous implications for the design of specific cysteine protease inhibitors. It shows that even though the catalytic machinery for stabilization of a tetrahedral transition state is present in papain, it will not necessarily be used by all compounds reacting with the enzyme and one must use caution in extrapolating the results obtained with a particular substrate to other systems. The precise geometry at the active site as well as the kinetics and mechanism of reaction must be considered individually for each compound under investigation. Studies are presently being carried out in our group to dissect out the requirements for utilization of the oxyanion hole interactions with various types of substrates and inhibitors.

Existence of an operational oxyanion hole in papain able to stabilize the transition-state complex by several kilocalories per mole gives further support to the concept that many enzymes work by complementing the changes in charge distribution occurring during the catalyzed reaction through electrostatic interactions (Warshel & Sussman, 1986; Warshel et al., 1989). For papain and cysteine proteases in general, catalysis involves a rearrangement of the charges from the thiolate–imidazolium residues of the ground state to the transition state. In this intermediate state, a significant portion of the negative charge of the thiolate anion is transferred to the carbonyl oxygen of the substrate. The presence of prealigned dipoles in the oxyanion hole can assist the charge transfer and stabilize the charged transition state, thus greatly accelerating the rate of reaction. In view of the fact that the Gln19 residue of papain is conserved in all known cysteine proteases, it is likely that the oxyanion hole interaction is an important feature of the catalytic mechanism of cysteine proteases in general.

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