A Classical Enzyme Active Center Motif Lacks Catalytic Competence until Modulated Electrostatically†

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ABSTRACT: The cysteine proteinase superfamily is a source of natural structural variants of value in the investigation of mechanism. It has long been considered axiomatic that catalytic competence of these enzymes mirrors the generation of the ubiquitous catalytic site imidazolium−thiolate ion pair. We here report definitive evidence from kinetic studies supported by electrostatic potential calculations, however, that at least for some of these enzymes the ion pair state which provides the nucleophilic and acid−base chemistry is essentially fully developed at low pH where the enzymes are inactive. Catalytic competence requires an additional protonic dissociation with a common pK_a value close to 4 possibly from the Glu50 cluster to control ion pair geometry. The pH dependence of the second-order rate constant (k) for the reactions of the catalytic site thiol groups with 4,4′-dipyrimidyl disulfide is shown to provide the pK_a values for the formation and deprotonation of the (Cys)-S^-/(His)-Im^+H ion pair state. Analogous study of the reactions with 2,2′-dipyridyl disulfide reveals other kinetically influential ionizations, and all of these pK_a values are compared with those observed in the pH dependence of k_{cat}/K_m for the catalyzed hydrolysis of *N*-acetylphenylalanylglycine 4-nitroanilide. The discrepancy between the pK_a value for ion pair formation and the common pK_a value close to 4 related to generation of catalytic activity is particularly marked for ficin (pK_a 2.49 ± 0.02) and caricain (pK_a 2.88 ± 0.02) but exists also for papain (pK_a 3.32 ± 0.01).

The cysteine proteinase superfamily (Barrett & Rawlings, 1991) which includes enzymes of biological and medical importance (Bazan & Fletterick, 1988; Mellor et al., 1993a; Scobie et al., 1994; Topham et al., 1994; Wilson et al., 1994; Gharbia et al., 1995) is a source of natural structural variants of value in the investigation of mechanism (Brocklehurst et al., 1987a; Brocklehurst, 1994; Taylor et al., 1994; Katerelos & Goodenough, 1996). Studies on the active center chemistry and catalytic mechanism of enzymes such as picornavirus cysteine proteinases (Bazan & Fletterick, 1988), the calpains (Mellor et al., 1993a), interleukin-1β converting enzyme (ICE)[1] (Wilson et al., 1994), the house dust mite allergen *Der p*1 (Scobie et al., 1994), and the gingivains, major virulence factors in destructive periodontal disease (Gharbia et al., 1995), rely heavily and build upon concepts developed from studies on cysteine proteinases of the papain family, notably papain itself (Brocklehurst et al., 1987a, 1997). A generally accepted concept of central importance in the catalytic mechanism of these enzymes is that catalytic competence develops synchronously with and consequent upon formation of the (Cys25)-S^-/(His159)-Im^+H (C^-H^+) ion pair state (papain numbering) by proton loss from (Cys25)-SH associated with pK_a *ca.* 4. This concept continues to be used as the basis of the interpretation of kinetic and protein engineering experiments (Ménard et al., 1991, 1995; Vernet et al., 1995). The thiolate−imidazolium ion pair contributed by the Cys25/His159/Asn175 catalytic site motif (analogous to the Ser/His/Asp triad of the serine proteinases) is the catalytic device that provides nucleophilic attack at the carbonyl carbon of the scissile bond of a substrate and general acid catalyzed assistance to leaving group departure from the resulting tetrahedral intermediate to produce the acyl enzyme (thiol ester) intermediate.

Doubt was cast on this concept, however, by the suggestion that, for papain, the pK_a value for the formation of the (Cys25)-S^-/(His159)-Im^+H ion pair state, determined by reactivity probe kinetics as 3.4 (Mellor et al., 1993b,c) and now refined to 3.32 ± 0.01 (see below), might not be identical with the value (*ca.* 4) determined for acyl enzyme formation in catalysis from the pH dependence of k_{cat}/K_m.

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[1] Abbreviations: 2PDS, 2,2′-dipyridyl disulfide; 4PMDS, 4,4′-dipyrimidyl disulfide; *N*-Ac-Phe-Gly-4NA, *N*-acetylphenylalanylglycine 4-nitroanilide; Bz-Gly, benzoylglycine; Bz-L-Arg, benzoyl-L-arginine; Cbz-L-Lys, carbobenzoxy-L-lysine; CH^+, (Cys25)-SH/(His159)-Im^+H; C^-H^+, (Cys25)-S^-/(His159)-Im^+H; C^-H, (Cys25)-S^-/(His159)-Im; DMF, *N*,*N*′-dimethylformamide; EDTA, ethylenediaminetetraacetic acid; ICE, interleukin-1β converting enzyme; PPΩ, papaya proteinase Ω (caricain); SF, stopped flow; v, initial rates.

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In the present work, the conclusion for papain has been placed on a secure footing by use of new, high quality kinetic data and by use of papain variants, caricain [papaya proteinase Ω, PPΩ (EC 3.4.22.30), a companion cysteine proteinase from *Carica papaya*] and ficin [(EC 3.4.22.3) from *Ficus glabrata*]. It was possible to demonstrate this important conclusion by a combination of pH-dependent kinetic studies on both catalysis and catalytic site reactivity using carefully designed time-dependent inhibitors as reactivity probes, and electrostatic potential calculations. The use of two papain variants demonstrates conclusively that the catalytic site (Cys25)-S^-/(His159)-Im^+H ion pairs already exist at low pH where these enzymes are catalytically inactive and that it is deprotonation of another site on the enzyme molecule (pK_a *ca.* 4) that produces catalytic competence. The much lower pK_a values for ion pair formation in caricain (2.88 ± 0.02) and in ficin (2.49 ± 0.02) than in papain (3.32 ± 0.01) and their consequent greater separation from the common pK_a value (*ca.* 4) characteristic of endowment of the ion pair with catalytic capability provide definitive evidence for an additional pH-dependent event without which the (Cys25)-S^-/(His159)-Im^+H ion pairs of these cysteine proteinases do not provide catalysis. This unexpected phenomenon, which is of central importance for the catalytic mechanism of these three cysteine proteinases, may be general for this enzyme family and possibly for the superfamily. In connection with the latter possibility, the active center architecture of ICE and the electrostatic field within 20 Å to which it is exposed are compared with the analogous features that typify the papain family of cysteine proteinases.

MATERIALS AND METHODS

Enzymes, Substrate, and Pyridyl Disulfide Reactivity Probes. Procedures for the purification of ficin (Malthouse & Brocklehurst, 1976), papain (Baines & Brocklehurst, 1979), and caricain (PPΩ; Topham et al., 1991) and for the synthesis, purification, and characterization of the substrate, *N*-Ac-Phe-Gly-4NA (Kowlessur et al., 1990), and 4,4′-dipyrimidyl disulfide, 4PMDS (Mellor et al., 1993b), have been described. 2,2′-Dipyridyl disulfide, 2PDS, was the Aldrich product twice recrystallized from petroleum ether (bp 60−80 °C) and had mp 58 °C [Marchwald et al. (1900) give mp 57−58 °C].

Stopped-Flow (SF) Kinetics of the Reactions of Papain, Caricain, and Ficin with 4,4′-Dipyrimidyl Disulfide (4PMDS) and with 2,2′-Dipyridyl Disulfide (2PDS). All reactions were carried out at 25 °C and $I = 0.1$ M in solutions containing 1 mM EDTA under pseudo-first-order conditions with [disulfide] >> [enzyme] using an Applied Photophysics SF.17MV stopped-flow spectrophotometer, kinetics workstation, and data acquisition and analysis software. Monochromator entrance and exit slit widths were set at 1 mm. The linear dependence of the first-order rate constant (k_{obs}) on [disulfide] established the adherence to overall second-order kinetics. At pH <7.0 the disulfide was in solution in the appropriate buffer, and the enzyme was in solution in 0.1 M KCl. In alkaline media the SF kinetic runs were carried out also with the enzyme in the buffer and the 2PDS in 0.1 M KCl, which demonstrated enzyme stability at pH ≤10.0 over at least 20 min. Reactions of the enzymes in alkaline media with 4PMDS, which is less stable than 2PDS toward alkaline hydrolysis, were carried out with the enzyme in the appropriate buffer, and SF records of progress curves were obtained within 5 min of introducing the enzyme into the buffer syringe. Reactions of 2PDS (2-Py-S-S-2-Py^+H ⇌ 2-Py-S-S-2-Py) were monitored at 343 nm with [enzyme] = 3−5 μM and [2PDS] = 50−230 μM. Release of the pyridine-2-thione was quantified by using $\Delta\epsilon_{343} = 8.08 \times 10^3/(1 + K_a/[H^+])$ M^{-1} cm^{-1}, where $pK_a = 9.8$ (Stuchbury et al., 1975). Reaction of 2PDS at pH 4.0 provides a convenient active site titration (Brocklehurst & Malthouse, 1981; Brocklehurst, 1996a), and values of [enzyme] refer to active site concentrations thus determined. Reactions of 4PMDS were monitored at the isosbestic point of the chromophoric product, pyrimidine-4-thione, 312 nm, with [enzyme] = 5−6 μM and [4PMDS] = 140−1420 μM. Concentrations of 2PDS and 4PMDS in appropriately diluted stock solutions were calculated from the infinity values of A_{343} and A_{312}, respectively, after thiolysis using an excess of 2-mercaptoethanol at pH 8.0.

Although unnecessary for the determination of first-order rate constants, the absorbance changes at 312 nm can be used at pH values ≥2 to calculate the concentration of product formed by using $\Delta\epsilon_{312} = 7763 - 317$ (the value of ϵ_{312} for 4PMDS at pH ≥2) = 7446 M^{-1} cm^{-1} (Mellor et al., 1993b,c). First-order rate constants (k_{obs}) were obtained by fitting the absorbance (A)−t data collected by the Acorn Archimedes microcomputer of the SF machine either to an equation for a single exponential process, i.e., $P_1e^{-P_2t} + P_3$, where $P_1 = A_\infty - A_0$, $P_2 = k_{obs}$, and $P_3 = A_\infty$, or to an equation for a single exponential with a zero-order component, i.e., $A = P_1e^{-P_2t} + P_3t + P_4$, where $P_1 = A_\infty - A_0$ for the single exponential component, $P_2 = k_{obs}$, P_3 = the slope of the zero-order component of the A *versus* t trace in s^{-1}, and P_4 = the extrapolated value of the ordinate intercept, i.e., A_∞ for the single exponential component. Reactions of 2PDS were evaluated by using the single exponential equation. Reactions of 4PMDS exhibited small but noticeable deviations from a single exponential process, particularly at pH values >7.5 [see also Mellor et al. (1993b,c)], and for these reactions the fit was improved by incorporation of a zero-order component. Values of second-order rate constants, k, were calculated from $k = k_{obs}/[\text{disulfide}]$, where the concentration of disulfide, the reagent in excess, was within the range over which the linear dependence of k_{obs} on [disulfide] had been established.

Kinetics of the Hydrolysis of N-Ac-Phe-Gly-4NA Catalyzed by Papain, Caricain, and Ficin. Initial rates (v) of release of 4-nitroaniline were measured at 410 nm using a Cary 1 spectrophotometer and quantified by using $\epsilon_{410} = 8.8 \times 10^3$ M^{-1} cm^{-1} (Erlanger et al., 1961). All kinetic runs were carried out at 8.3% (v/v) of *N*,*N*′-dimethylformamide (DMF) with [enzyme] = 1.0−2.5 μM and [S] = 25−100 μM in a 3.0 mL reaction volume such that the conditions $[E]_T << [S] << K_m$ were obeyed. Values of $[E]_T$ were determined by titration with 2PDS, and values of k_{cat}/K_m at each pH were calculated from $k_{cat}/K_m = v/[E]_T[S]$. Stock solutions of the substrate (1.5 mM) were prepared in 50% (v/v) aqueous DMF. Concentrations were calculated from infinity values of A_{410} of appropriately diluted solutions following complete enzyme-catalyzed or alkaline hydrolysis.

Computer Evaluation of the pH-Dependent Kinetic Data. pH-dependent kinetic studies of the type reported here involving a multiplicity of reactive states require a rapid method of evaluating a series of kinetic models differing in the number and reactivity of the reactive states [see Brocklehurst (1994, 1996b)]. These were evaluated by using a multitasking application program (SKETCHER) written in

ANSI C running under RISCOS on an Acorn Archimedes microcomputer (Brocklehurst et al., 1990; Topham et al., 1991), which was developed from an earlier program (QUATRO) written in BBC BASIC for use with a BBC model B (Brocklehurst & Brocklehurst, 1988). Rate equations for reactions in a variety of protonic states were written down and generated within the Archimedes by using the simple general expression:

$$k = \sum_{j=1}^{n} \frac{A_i}{(1 + \sum_{j=1}^{n} B_{i,j})}$$

This relates an experimentally determined pH-dependent rate constant, k, and macroscopic acid dissociation constants, K, where n is the number of reactive protonic states; the numerator A_i is a generalized pH-independent rate constant $\tilde{k}_{XH_{i-1}}$, where i, which may take values from 1 to n, specifies the particular protonic state. The denominator provides for the pH-dependent variation in each of the contributions to k from reaction in a particular protonic state, i.e., reaction associated with an individual pH-independent rate constant ($\tilde{k}$). Each term generated by j in the denominator comprises a product of $[H^+]$ and one or more acid dissociation constants, each raised to a particular power. The construction of specific rate equations for particular kinetic models involves determining expressions for $B_{i,j}$ by using the two information matrices described by Brocklehurst et al. (1990) and Topham et al. (1991). SKETCHER permits rapid estimation of characterizing parameters in the generic set of equations for the various models by means of interactive manipulation of calculated curves. Values of the parameters thus obtained are used as provisional estimates for analysis by weighted nonlinear regression performed by using the AR computer program from the statistical software package BMDP (Dixon et al., 1988) or the nonlinear regression program in SIGMAPLOT 2.01 (Jandel Scientific) and an IBM 150 MHz PC. In the present work an error structure of constant relative error was assumed and weighting factors were inversely proportional to k^2 or to $(k_{cat}/K_m)^2$. pH$-k$ data and the associated theoretical curves were displayed by using SIGMAPLOT 2.01 (Jandel Scientific), a Pentium 120 or 150 MHz IBM compatible PC, and a Hewlett-Packard LaserJet 5P printer.

The possibility that some data sets might require relatively complex kinetic models, in some cases with closely spaced pK_a values, was investigated statistically by using the procedure described by Mannervik (1982). Thus two models, j and k, are considered for a particular data set where model k (with p_k parameters determined by the weighted nonlinear regression procedure) is related to the simpler model j (with p_j such parameters). The significance of the postulated improvement of the fit to the data consequent upon addition of the ($p_k - p_j$) new parameters is evaluated by comparison of the value of the quotient $F_{(pk-pj,\ n-pk)} = (SS_j - SS_k)(n - p_k)/(p_k - p_j)SS_k$ (where SS_j and SS_k are the weighted residual sums of squares and n is the number of data points) with the value of the F-statistic, $F_{\alpha(pk-pj,\ n-pk)}$ at an appropriate level of probability of difference by chance (0.05), α, obtained by using the MINITAB software package.

Electrostatic Potential Calculations. The crystal structures of papain (9pap.pdb; Kamphuis et al., 1984), caricain (1ppo.pdb; Pickersgill et al., 1991), and interleukin-1β converting enzyme (ICE; 1ice.pdb; Wilson et al., 1994) with the blocking group bonded to the catalytic site Cys S_γ removed were used as the basis for modeling. The buried water molecules (9 in 9pap and 7 in 1ppo) were retained (see the Discussion section). The initial models were established by the techniques described previously (Plou et al., 1996). In particular, use was made of the extended-atom CHARMM force field (Brooks et al., 1983), polar hydrogen atoms were added using the HBUILD functionality (Brunger & Karplus, 1988) of CHARMM, and solvent was represented using a modified form of the TIP3 model (Jorgensen et al., 1983). Then values of ΔG, the free energy change for the formation of the (Cys)-S^-/(His)-Im^+H (C^-H^+) ion pair, were calculated as the energies required to place a unit negative charge at S_γ by solving the linearized Poisson−Boltzmann equation using finite difference methods and the program University of Houston Brownian Dynamics (UHBD, version 5.1; Madura et al., 1995). The following fractional charge distribution was used: each Lys and the N-terminus (+1); each Arg (+0.5 on each terminal N); each His ring N (+0.5); the C-terminus and each Asp and Glu were considered in turn to be un-ionized, ionized, and half-ionized. The dielectric constants of protein and solvent were set to 2 (or 20) and 78, respectively. The other experimental conditions were simulated using an ionic concentration of 100 mM, a 2 Å Stern layer around a Richards surface of accessibility (Antosiewicz et al., 1994), CHARMM atomic radii (Brooks et al., 1983), and a temperature of 300 K. The Richards accessible surface was generated for a probe of radius 1.4 Å, and this delineated the regions of the two dielectrics (Antosiewicz et al., 1994). Finite difference focusing and dielectric boundary smoothing (Gilson et al., 1993) were used such that a 300 Å × 300 Å × 300 Å cubic grid was partitioned into 2 Å × 2 Å × 2 Å blocks. Subsequently, this grid was decreased in size to 112.5 Å × 112.5 Å × 112.5 Å and partitioned into 0.75 Å × 0.75 Å × 0.75 Å blocks. Finally, this process was extended to produce a grid of 15 Å × 15 Å × 15 Å partitioned into 0.1 Å × 0.1 Å × 0.1 Å blocks. The initial grid accommodates the protein (longest length 50 Å) and a surrounding region of length 125 Å on either side of the protein. The energy required to place a negative charge at S_γ of Cys25 was calculated as −1.0 × the net potential generated at this site by the charges surrounding it. The pK_a value for C^-H^+ formation in caricain was calculated using the pK_a for papain determined experimentally (3.32), the calculated value of $\Delta\Delta G = \Delta G_{caricain} - \Delta G_{papain}$, and $\Delta pK_a = \Delta\Delta G/1.3645$ [see Plou et al. (1996)].

To investigate the contributions to ΔG from individual charged groups in the protein, the electrostatic potential at S_γ of Cys25 was computed with unit charge (positive or negative as appropriate) deleted on each ionizable site in turn (i.e., the N-terminus, the side chain of the Arg, His, and Lys residues, the C-terminus, and the side chains of the Asp and Glu residues) with the other ionizable sites of the same charge type charged. When individual cationic sites were investigated, all of the potentially anionic sites were assumed to be uncharged. It had already been demonstrated that the ΔpK_a value for ion pair formation is not affected by the charge status of the carboxy groups of the proteins.

To attempt to locate the source of the kinetically influential ionization with pK_a *ca.* 4 common to papain and caricain (presumed to be a carboxyl group), all cationic sites were assigned charges of +1, S_γ of Cys25 was assigned a charge

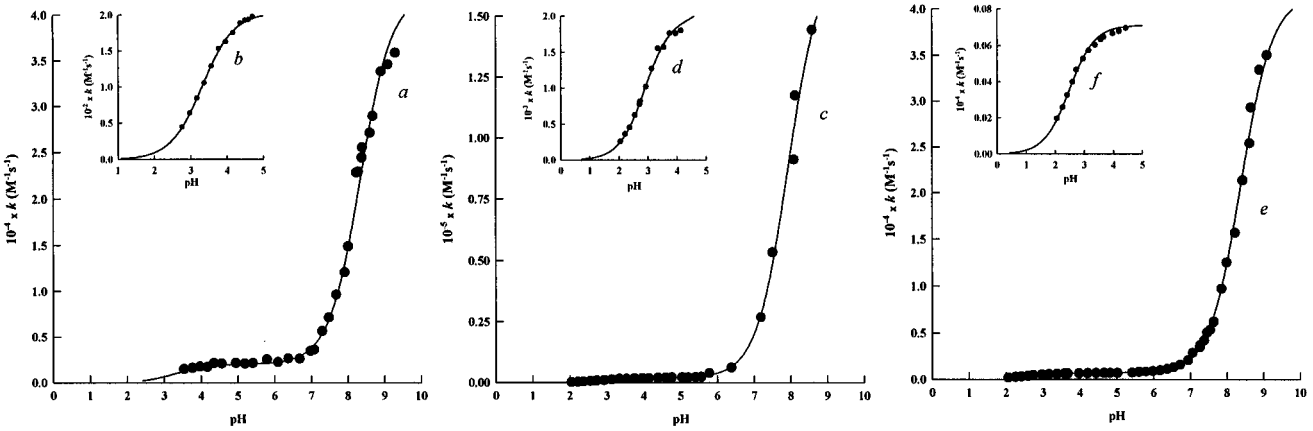
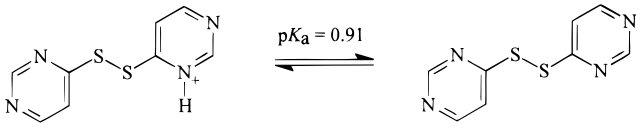


FIGURE 1: pH dependence of the second-order rate constant (k) for the reactions at 25 °C and I 0.1 M of 4,4′-dipyrimidyl disulfide (4PMDS) (a and b) with papain, (c and d) with caricain, and (e and f) with ficin. The points are experimental and the lines are theoretical for the pH-dependent rate equation for two reactive protonic states, $k = \tilde{k}_1/(1 + [H^+]/K_1 + K_2/[H^+]) + \tilde{k}_2/(1 + [H^+]^2/K_1K_2 + [H^+]/K_2)$, and the following values of the characterizing parameters, with standard errors in parentheses, determined by weighted nonlinear regression, with values of the pH-independent rate constants, $\tilde{k}$, in M^{-1} s^{-1}: for the papain reaction, pK_1 = 3.32 (±0.01), pK_2 = 8.34 (±0.04), $\tilde{k}_1 = 2.03 \times 10^3$ ($\pm 0.02 \times 10^3$), $\tilde{k}_2 = 4.2 \times 10^4$ ($\pm 0.2 \times 10^4$); for the caricain reaction, pK_1 = 2.88 (±0.02), pK_2 = 7.93 (±0.05), $\tilde{k}_1 = 1.96 \times 10^3$ ($\pm 0.05 \times 10^3$), $\tilde{k}_2 = 1.73 \times 10^5$ ($\pm 0.01 \times 10^5$); for the ficin reaction, pK_1 = 2.49 (±0.02), pK_2 = 8.39 (±0.02), $\tilde{k}_1 = 7.1 \times 10^2$ ($\pm 0.1 \times 10^2$), $\tilde{k}_2 = 4.2 \times 10^4$ ($\pm 0.1 \times 10^4$). For these reactions, values of pK_1 characterize the formation of the catalytic site C^-H^+ ion pair states from CH^+ and values of pK_2 characterize proton loss from the ion pair states to form the "uncomplicated" thiolate anions, C^-H.

Scheme 1: Two Protonation States (**1** and **2**) of 4,4′-Dipyrimidyl Disulfide (4PMDS)[a]



1 **2**

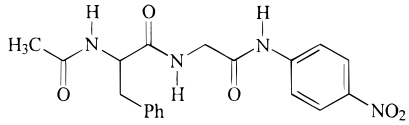
[a] The pK_a value of **1** (0.91) is sufficiently low that reaction with the thiol groups of papain, caricain, and ficin (pK_a values of 3.32, 2.88, and 2.49, respectively) is essentially confined to reaction of the unprotonated form **2**.

of −1, and the C-terminus and the side chains of the Asp and Glu residues were assumed to be uncharged. The interaction energy between the C^-H^+ ion pair and each carboxylate anion in the presence of the various cationic groups was calculated as the energy required to place a charge of −1 on each carboxyl group in turn (a charge of −0.5 on each of the carboxyl O atoms). An analogous procedure was used to investigate electrostatic influences in ICE with S_γ of the catalytic site Cys285 assigned a charge of −1.

RESULTS

Determination of the pK_a Values of the Catalytic Site (Cys)-S$^-$/(His)-Im$^+$H Ion Pairs of Papain, Caricain, and Ficin. The pK_a values characteristic of the formation ($CH^+ \rightleftharpoons C^-H^+$) and subsequent deprotonation ($C^-H^+ \rightleftharpoons C^-H$) of the (Cys)-S$^-$/(His)-Im$^+$H ion pair states of the catalytic sites of papain (3.32 ± 0.01 and 8.34 ± 0.04), caricain (2.88 ± 0.02 and 7.93 ± 0.05), and ficin (2.49 ± 0.02 and 8.39 ± 0.02) were determined by analysis of the pH dependence of the second-order rate constant (k) (Figure 1) for their reactions with 4PMDS (**1** ⇌ **2**, Scheme 1). These values are similar to the provisional estimates obtained by using SKETCHER: for papain, 3.40 and 8.30 (Mellor et al., 1993b,c); for caricain, 2.90 and 8.20; for ficin, 2.46 and 8.47. In all three cases these pH−k profiles are of double sigmoidal form, k increasing in two stages with increase in pH. The simplicity of these profiles readily permits the determination of the characterizing pK_a values for C^-H^+ ion pair formation and deprotonation to the "uncomplicated" thiolate anion, C^-H, respectively (see the Discussion section).

Detection and Characterization of the Kinetically Influential Ionizations of Papain, Caricain, and Ficin Associated with Catalytic Competence. The pH dependences of k_{cat}/K_m for the hydrolysis of the substrate *N*-Ac-Phe-Gly-4NA (**3**) catalyzed by these enzymes (Figure 2a,c,e) taken in



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conjunction with the data provided by Figure 1 are remarkable in demonstrating that C^-H^+ ion pair formation, unexpectedly, does not provide catalytic competence. Thus, a model in which the traditional view that C^-H^+ formation is both necessary and sufficient for generation of catalytic competence is assumed does not provide a fit to the data, particularly in acidic media, as demonstrated in Figure 2b,d,f. Initial evaluation of the data in Figure 2 using SKETCHER suggested the generation of low catalytic activity consequent upon formation of the C^-H^+ ion pair ($\tilde{k}_{cat1}/\tilde{K}_{m1}$) with substantial enhancement of activity consequent upon further protonic dissociation (with pK_a *ca.* 4, $\tilde{k}_{cat2}/\tilde{K}_{m2}$). The sets of values of $\tilde{k}_{cat1}/\tilde{K}_{m1}$ and $\tilde{k}_{cat2}/\tilde{K}_{m2}$ (in M^{-1} s^{-1}) thus obtained are as follows: for papain, 10, 1.95×10^3; for caricain, 50, 4.00×10^2; for ficin, 1.0, 2.25×10^2. Statistical analysis (Mannervik, 1982) of fits to the pH *versus* k_{cat}/K_m data obtained by weighted nonlinear regression using models with and without the catalytically active C^-H^+ ion pair in the absence of protonic dissociation with pK_a *ca.* 4, as described in the Materials and Methods section, demonstrated the sufficiency of the simpler model used as the basis of the fits shown in Figure 2a,c,e. Thus for these three enzymes, there is no evidence for catalytic competence consequent upon C^-H^+ ion pair formation before the protonic dissociation with pK_a *ca.* 4 (the electrostatic switch, see the Discussion section) has occurred. A minor characteristic of the three pH *versus*

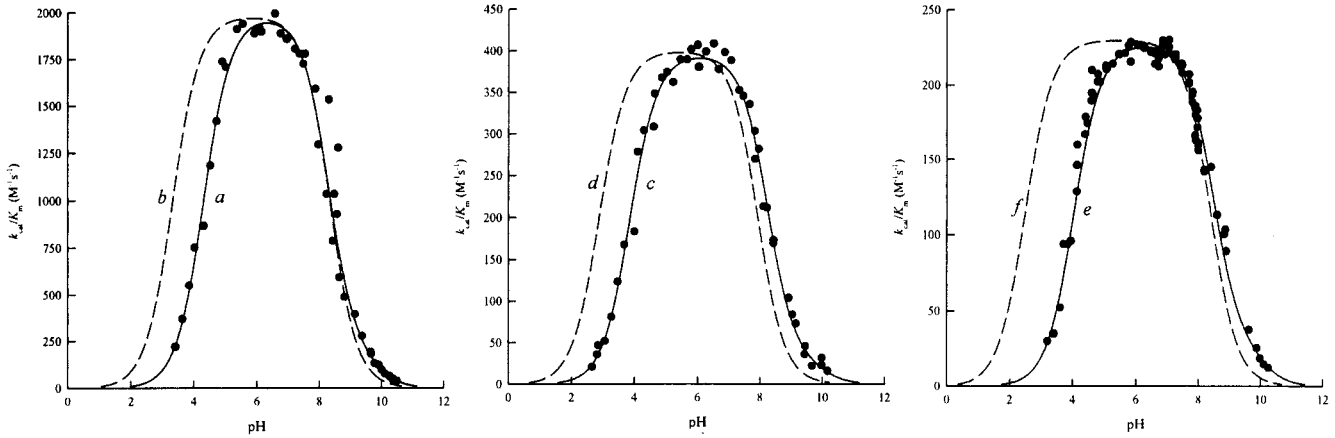


FIGURE 2: pH dependence of k_{cat}/K_m for the hydrolysis of *N*-Ac-Phe-Gly-4NA at 25 °C and *I* 0.1 M catalyzed (a and b) by papain, (c and d) by caricain, (e and f) by ficin. The points were determined experimentally as described in the Materials and Methods section. The broken lines are theoretical for the pH-dependent rate equation for one reactive protonic state bounded by two unreactive protonic states, $k_{cat}/K_m = \tilde{k}_{cat}/\tilde{K}_m/(1 + [H^+]/K_1 + K_2/[H^+])$, and the following values of the characterizing parameters (with values of $\tilde{k}_{cat}/\tilde{K}_m$ in M^{-1} s^{-1}): for (b) the papain-catalyzed reaction, $pK_1 = 3.32$, $pK_2 = 8.34$, $\tilde{k}_{cat}/\tilde{K}_m = 1.98 \times 10^3$; for (d) the caricain-catalyzed reaction, $pK_1 = 2.88$, $pK_2 = 7.93$, $\tilde{k}_{cat}/\tilde{K}_m = 4.00 \times 10^2$; for (f) the ficin-catalyzed reaction, $pK_1 = 2.49$, $pK_2 = 8.39$, $\tilde{k}_{cat}/\tilde{K}_m = 2.30 \times 10^2$. These lines which correspond to the situation in which catalytic competence is assumed to develop synchronously with C^-H^+ ion pair formation from CH^+ do not fit the data, particularly on the acid limb of the profile. The continuous lines are theoretical for the pH-dependent rate equation for activity in two protonic states bounded by two inactive states, $k_{cat}/K_m = \tilde{k}_{cat_1}/\tilde{K}_{m_1}/(1 + [H^+]/K_1 + K_2/[H^+] + K_1K_2/[H^+]^2) + \tilde{k}_{cat_2}/\tilde{K}_{m_2}/(1 + [H^+]^2/K_1K_2 + [H^+]/K_2 + K_3/[H^+])$, and the following values of the characterizing parameters with standard errors in parentheses for the parameters determined by weighted nonlinear regression and with values of $\tilde{k}_{cat}/\tilde{K}_m$ in M^{-1} s^{-1}: for (a) papain, $pK_1 = 4.30$ (±0.04), $pK_2 = 8.28$ (±0.05), $pK_3 = 9.80$, $\tilde{k}_{cat_1}/\tilde{K}_{m_1} = 1.98 \times 10^3$ (±0.07 × 10^3), $\tilde{k}_{cat_2}/\tilde{K}_{m_2} = 2.18 \times 10^2$ (±0.11 × 10^2); for (b) caricain, $pK_1 = 3.85$ (±0.03), $pK_2 = 8.24$ (±0.06), $pK_3 = 10.00$, $\tilde{k}_{cat_1}/\tilde{K}_{m_1} = 4.00 \times 10^2$ (±0.1 × 10^2), $\tilde{k}_{cat_2}/\tilde{K}_{m_2} = 32$ (±3); for (c) ficin, $pK_1 = 4.03$ (±0.02), $pK_2 = 8.45$ (±0.04), $pK_3 = 10.00$, $\tilde{k}_{cat_1}/\tilde{K}_{m_1} = 2.30 \times 10^2$ (±0.03 × 10^2), $\tilde{k}_{cat_2}/\tilde{K}_{m_2} = 32$ (±2). The continuous lines b, d, and f correspond to the dependence of the development of catalytic competence consequent upon deprotonation with pK_a *ca.* 4 (the electrostatic switch; see the text). Use of a more complex model with three reactive protonic states which assumes that development of some catalytic competence consequent upon formation of the C^-H^+ ion pair at lower pH values (across pK_a values 3.32, 2.88, and 2.49 for papain, caricain, and ficin, respectively) does not produce a statistically significant improvement of the fit (see the text).

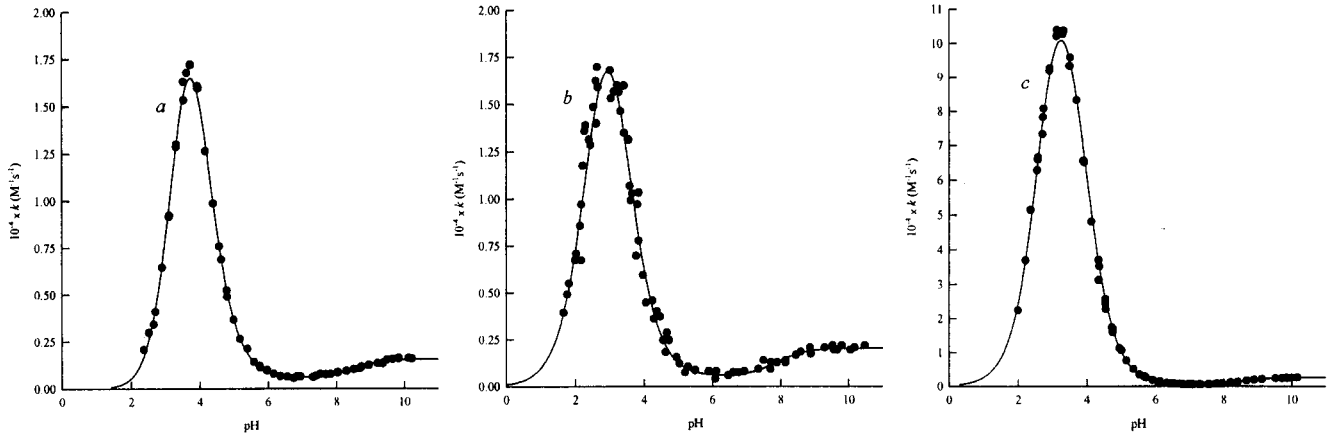
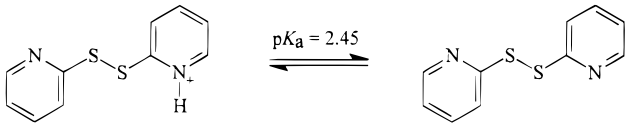


FIGURE 3: pH dependence of the second-order rate constant (*k*) for the reactions at 25 °C and *I* 0.1 M of 2,2′-dipyridyl disulfide (2PDS) (a) with papain, (b) with caricain, and (c) with ficin. The points are experimental and the lines are theoretical for the pH-dependent rate equation for four reactive protonic states, $k = \tilde{k}_1/(1 + [H^+]/K_1 + K_2/[H^+] + K_2K_3/[H^+]^2 + K_2K_3K_4/[H^+]^3) + \tilde{k}_2/(1 + [H^+]^2/K_1K_2 + [H^+]/K_2 + K_3/[H^+] + K_3K_4/[H^+]^2 + \tilde{k}_3/(1 + [H^+]^3/K_1K_2K_3 + [H^+]^2/K_2K_3 + [H^+]/K_3 + K_4/[H^+]) + \tilde{k}_4/(1 + [H^+]^4/K_1K_2K_3K_4 + [H^+]^3/K_2K_3K_4 + [H^+]^2/K_3K_4 + [H^+]/K_4)$, and the following values of the characterizing parameters with standard errors in parentheses for the parameters determined by weighted nonlinear regression and with values of the pH-independent rate constants, $\tilde{k}$, in M^{-1} s^{-1}: for (a) the papain reaction, $pK_1 = 2.40$, $pK_2 = 3.39$ (±0.08), $pK_3 = 4.03$ (±0.05), $pK_4 = 8.48$ (±0.08), $\tilde{k}_1 = 6.0 \times 10^2$ (±5.0 × 10^2), $\tilde{k}_2 = 3.21 \times 10^4$ (±0.28 × 10^4), $\tilde{k}_3 = 5.48 \times 10^2$ (±0.17 × 10^2), $\tilde{k}_4 = 1.54 \times 10^3$ (±0.05 × 10^3); for (b) the caricain reaction, $pK_1 = 2.44$ (±0.08), $pK_2 = 3.43$ (±0.07), $pK_3 = 7.88$ (±0.12), $\tilde{k}_1 = 2.74 \times 10^4$ (±0.29 × 10^4), $\tilde{k}_2 = 5.00 \times 10^2$ (±0.40 × 10^2), $\tilde{k}_3 = 2.00 \times 10^3$ (±0.10 × 10^3); for (c) the ficin reaction, $pK_1 = 2.17$, $pK_2 = 2.77$ (±0.08), $pK_3 = 3.84$ (±0.02), $pK_4 = 8.57$ (±0.04), $\tilde{k}_1 = 3.95 \times 10^4$ (±0.54 × 10^4), $\tilde{k}_2 = 1.50 \times 10^5$ (±0.06 × 10^5), $\tilde{k}_3 = 3.88 \times 10^2$ (±0.12 × 10^2), $\tilde{k}_4 = 2.59 \times 10^3$ (±0.06 × 10^3). The values of pK_1 (2.40 and 2.17) used to fit the data for the reactions of papain and ficin, respectively, differ significantly from those of $2PDS^+H$ (2.45) determined spectroscopically as a result of their proximity to and consequent perturbation by mixing with those of C^-H^+ ion pair formation.

k_{cat}/K_m profiles (Figure 2a,c,e) is the need for protonic dissociation with pK_a *ca.* 10 in addition to conversion of C^-H^+ to C^-H (pK_a values: for papain, 8.28 ± 0.05; for caricain, 8.24 ± 0.06; for ficin, 8.45 ± 0.04) to provide a good fit to the data in alkaline media. In all three cases, the better fit of the model with the additional pK_a value is evidenced by values of $F/F_\alpha > 1$ (see the Materials and Methods section), the values of this ratio being the following: for papain, 17.1; for caricain, 9.7; for ficin, 40.4.

The Kinetically Influential Ionizations of Papain, Caricain, and Ficin Detected by Using 2,2′-Dipyridyl Disulfide (2PDS) as a Reactivity Probe. The pH−*k* profiles (Figure 3) for the reactions of all three enzymes with the 2-pyridyl disulfide probe (**4** ⇌ **5**, Scheme 2) are markedly different in shape

Scheme 2: Two Protonation States (**4** and **5**) of 2,2′-Dipyridyl Disulfide (2PDS)[a]



4 **5**

[a] The pK_a value of **4** (2.45) is sufficiently close to those of the thiol groups of papain, caricain, and ficin (see Scheme 1 legend) that significant concentrations of the more reactive form **4** and C^-H^+ ion pair coexist. This leads to the striking rate maxima in acidic media (Figure 3) in the reaction of 2PDS, which are without analogue in reactions of 4PMDS (Figure 1).

from those of the corresponding reactions with the pyrimidyl disulfide probe (**2**) (Figure 1). The former profiles (Figure 3) contain striking rate maxima in acidic media, which are absent in the pH−*k* profiles in Figure 1, and for the reactions of papain and ficin, evidence of major additional kinetically influential ionizations with pK_a values *ca.* 4 (4.03 ± 0.05 for papain; 3.84 ± 0.02 for ficin). It is noteworthy that these values are similar to, but somewhat lower than, the pK_a values characteristic of the development of catalytic competence (k_{cat}/K_m) in Figure 2a,e, where the values are as follows: for papain, 4.30 ± 0.04, and for ficin, 4.03 ± 0.02. The variation in the value of pK_1 in Figure 3 which derives from the pK_a value of $2PDS^+H$ determined by spectroscopic titration as 2.45, is the result of perturbation by mixing with the pK_a for C^-H^+ ion pair formation, which is itself perturbed in the opposite direction. If K_e is an acid dissociation constant of the enzyme (for the formation of the C^-H^+ ion pair state in the present context) and K_r is the acid dissociation constant of the reactivity probe ($10^{-2.45}$ for $2PDS^+H$), K_1 (the first experimentally observed macroscopic acid dissociation constant) is given by $K_1 = 10^{-pK_e} + 10^{-pK_r}$ (Malthouse & Brocklehurst, 1976), where the values of pK_e determined by reactivity probe kinetics using 4PMDS are as follows: for papain, 3.32 ± 0.01, and for ficin, 2.49 ± 0.02. Thus for the reactions with $2PDS^+H$, the perturbed values of the pK_a of $2PDS^+H$ (pK_1) are as follows: for the papain reaction, $-\log(10^{-3.32} + 10^{-2.45}) = 2.4$, and for the ficin reaction, $-\log(10^{-2.49} + 10^{-2.45}) = 2.17$. The values of p$K_2$ (the perturbed pK_a for C^-H^+ formation) calculated by using $K_2 = K_eK_r/(K_e + K_r)$ are in good agreement with the values obtained by weighted nonlinear regression of the data in Figure 3 (given in parentheses): for papain, 3.38 (3.39 ± 0.08), and for ficin, 2.77 (2.77 ± 0.08). The need for the three pK_as in each of the bell-shaped components of Figure 3a,c to provide a good fit to the data is indicated by the values of F/F_α greater than unity when the fits using the three pK_a model are compared to those using a two pK_a model: for the papain reaction, $F/F_\alpha = 3.1$, and for the ficin reaction, $F/F_\alpha = 1.4$.

For the caricain reaction use of a three pK_a model for the bell-shaped component of the pH−*k* profile is not supported by the statistical evidence ($F/F_\alpha = 0.1$). The simpler model (two pK_as in the bell and three pK_as for the complete pH−*k* profile) is characterized by the pK_a of $2PDS^+H$ (pK_1) and another pK_a (pK_2) of 3.43 ± 0.07. The electrostatic switch (pK_a *ca.* 4) appears to be absent or of minor importance in the reaction of caricain with $2PDS^+H$. It is possible that pK_2 (3.43) could be the unresolved resultant of the overlap of the pK_a for C^-H^+ formation and that of the electrostatic switch with pK_a *ca.* 4 [see Brocklehurst et al. (1983) and Brocklehurst (1994) for a discussion of curves with overlapping kinetically influential pK_a values and circumstances in which they are difficult to distinguish from a single ionization curve].

Table 1: Free Energy Changes and Calculated and Experimentally Determined pK_a Values for C^-H^+ Ion Pair Formation in the Catalytic Sites of Three Cysteine Proteinases

enzyme	ΔG (kcal mol^{-1})[d]	$\Delta\Delta G$ (kcal mol^{-1})[e]	ΔpK_a[f]	pK_a[g] (calc)	pK_a[h] (exptl)
papain[a]	−14.81				3.32
caricain[b]	−15.67	−0.86	−0.63 (−0.44)	2.69 (2.88)	2.88
ficin[c]					2.49

[a] Brookhaven Protein Data Bank (PDB) entry 9pap. [b] PDB entry 1ppo. [c] 3D structure not available. [d] Values of the change in free energy (ΔG) for the formation of C^-H^+ from CH^+ calculated as the energy required to place a unit negative charge at the sulfur atom of the catalytic site cysteine residue assuming a protein dielectric constant of 2; for further details see the text. [e] $\Delta\Delta G = \Delta G_{caricain} - \Delta G_{papain}$. [f] $\Delta pK_a = \Delta\Delta G/1.3645 = -0.63$; the calculated value of ΔpK_a, −0.63, was obtained when the buried water molecules were retained in the protein structures; this value provides a calculated pK_a value for C^-H^+ formation in caricain that is 93% of the value determined experimentally; in the absence of these water molecules the calculated and experimentally determined values of the pK_a are identical (2.88; $\Delta pK_a = -0.44$). [g] pK_a(calc) for caricain = pK_a(exptl) for papain − 0.63. [h] Determined by reactivity probe kinetics using 4PMDS.

Calculation of the Difference in the pK_a Values for Formation of the C^-H^+ Ion Pair State from the CH^+ State in the Catalytic Sites of Papain and Caricain. Calculated values of ΔG for C^-H^+ formation in the two cysteine proteinases, with the buried water molecules retained and assuming a protein dielectric constant of 2, and the corresponding calculated values of $\Delta\Delta G$ and ΔpK_a are shown in Table 1. Given the pK_a value for C^-H^+ formation in papain determined experimentally by reactivity probe kinetics using 4PMDS as 3.32, the pK_a value for the analogous process in caricain calculated as 2.69 is in reasonable agreement with the value determined experimentally as 2.88.

Contributions of Individual Ionic Groups to the Difference in Free Energy Change and Hence in the pK_a Value for the Formation of C^-H^+ from CH^+ in Papain and Caricain: Possible Major Roles for Lys64, Lys137, His18, and His61. The contributions of each ionic side chain and of the N- and C-termini in each enzyme to the energy required to place a unit negative charge at S_γ of CH^+ are shown in Table 2. None of the anionic sites makes a large contribution uniquely to only one of the enzymes. The largest contribution in caricain is from Arg17, but there is a similar contribution from Lys17 in papain. The largest contributors in caricain that are without analogues in papain are Lys64, Lys137, His18, and His61.

Electrostatic Interaction Energies between Individual Carboxylate Anions and the C^-H^+ Ion Pairs of Papain, Caricain, and ICE: the Glu50 Side Chain as a Candidate for the Kinetically Influential Ionizing Group with pK_a ca. 4 in Papain and Caricain and Asp185 as a Candidate for an Analogous Group in ICE. Values of these interaction energies for papain and caricain are shown in Table 3 and values of the analogous interaction energies in ICE are shown in Table 4. The ionic group common to papain and caricain closest to the C^-H^+ ion pair with large values of the interaction energy is the side chain of Glu50. Our current hypothesis is that this is the favored candidate for the kinetically influential ionization with pK_a *ca.* 4 in both papain

Table 2: Contributions of Individual Ionic Side Chains in Papain and Caricain to the Free Energy Change for the Formation of the C^-H^+ State of the Catalytic Site from the CH^+ State (ΔG)

residue[a] contributing charge *		$-\Delta\Delta G^b$ (kcal mol^{-1})		av distance[c] from centroid of C^-H^+ (Å)	
papain	caricain	papain	caricain	papain	caricain
N-terminus	N-terminus	0.01	0.01	30.1	29.6
R8	R8	0.09	0.07	19.7	20.0
K17	R17	0.72	0.96	15.4	15.0
R41	R41	0.02	0.03	27.2	26.4
R58	R58	0.10	0.05	19.5	21.8
R59	R59	0.16	0.17	19.2	18.5
R83	R83	0.49	0.49	15.1	14.8
R93	K91	0.10	0.04	20.9	20.6
R96	R96	0.16	0.12	20.6	21.3
R98	K98	0.02	0.02	27.5	26.1
R111		0.03		25.3	
	R112		0.14		19.1
K139	R139	0.06	0.06	19.5	19.1
R145	K145	0.03	0.04	21.7	19.5
R188	R192	0.04	0.06	22.1	21.3
R191	R195	0.04	0.06	24.6	23.9
	K9		0.02		25.1
K10	K10	0.03	0.04	25.4	25.7
K39	K39	0.02	0.03	27.4	26.5
	K44		0.01		30.4
	K64		0.36		11.9
	K77		0.03		24.3
	K85		0.02		28.4
K100		0.04		24.0	
K106	K106	0.02	0.01	27.2	24.0
	K127		0.04		24.4
	K137		0.47		10.7
K156	K156	0.09	0.15	16.4	15.1
	K168		0.08		28.7
	K172		0.01		25.3
K174	K178	1.16	1.18	14.1	13.7
	K188		0.03		21.2
K190	K194	0.02	0.02	25.0	24.1
	K208		0.19		15.5
K211	K215	0.03	0.06	26.9	25.5
	H18		0.41		11.4
	H61		0.41		13.4
H81	H81	0.11	0.11	24.2	23.7
D6	D6	0.12	0.13	21.4	21.0
D55	D55	0.53	0.49	17.5	17.4
D57	E57	0.95	0.80	14.6	14.9
D108		0.03		27.2	
D140		0.06		17.7	
D158	D158	1.20	1.38	7.1	7.2
E3	E3	0.01	0.01	31.1	30.1
E35	E35	0.94	0.96	16.0	15.7
E47	E47	0.15	0.10	19.0	19.3
E50	E50	1.81	1.83	12.9	12.5
E52	E52	0.32	0.39	20.3	20.0
	E73		0.13		19.2
E89	K89	0.11	0.05	19.7	21.2
E99		0.02		27.6	
E118	E118	0.09	0.05	22.2	23.8
E135	E135	0.28	0.31	12.9	12.8
	E150		0.03		22.7
E183	E187	0.10	0.11	19.2	18.9
C-terminus	C-terminus	0.01	0.01	29.8	29.5

[a] Noncharged residues in equivalent positions are not shown; the prime candidates for the origin of the electrostatic perturbation of C^-H^+ are underlined. [b] For the cationic residues and the N-terminus $\Delta\Delta G = \Delta G_{(\text{all positive charges})} - \Delta G_{(\text{with charge * deleted})}$; values of ΔG were calculated as the energy required to place a unit negative charge at S_γ of the catalytic site cysteine residue; a protein dielectric constant of 2 was assumed, and the buried water molecules were retained; $\Delta G_{(\text{all positive charges})} = -14.81$ kcal mol^{-1} for papain and -15.67 kcal mol^{-1} for caricain; for the anionic residue and the C-terminus $\Delta\Delta G = \Delta G_{(\text{all charges})} - \Delta G_{(\text{with charge * deleted})}$; $\Delta G_{(\text{all charges})} = -8.09$ kcal mol^{-1} for papain and -8.93 kcal mol^{-1} for caricain. [c] Distances: for carboxylate side chains the distances are the averages of those to the two oxygen atoms; for Arg, the averages of the distances to the terminal nitrogen atoms are shown; for Lys, the distances are those to N_ϵ; for His, the averages of the distances to the two nitrogen atoms are shown.

Table 3: Electrostatic Energies of Interaction (ΔG) between the C^-H^+ Ion Pairs of the Catalytic Sites of Papain and Caricain and Individual Carboxylate Anions in the Presence of All of the Positive Charges on the Protein

residue contributing the carboxylate anion[a]		$-\Delta G^b$ (kcal mol^{-1})	
papain	caricain	papain	caricain
D6	D6	13.6	16.8
D55	D55	26.7	12.5
D108		6.6	
D140		1.0	
D158	D158	2.6	17.1
E3	E3	1.2	3.5
E35	E35	40.4	53.2
E47	E47	18.3	10.7
E50	E50	37.5	52.7
E52	E52	27.2	25.8
D57	E57	8.6	10.8
	E73		8.8
E99		13.9	
E118	E118	8.0	7.0
E135	E135	5.7	8.3
	E150		15.7
E183	E187	8.6	12.6
C-terminus	C-terminus	3.1	3.1

[a] Noncharged residues in equivalent positions are not shown; the prime candidate for the electrostatic switches (Glu50), identified by the relatively large value of the interaction energy and their proximity to C^-H^+ in both enzymes (12.9 Å in papain and 12.5 Å in caricain), and another potential candidate (Glu35) are underlined; the distances of the carboxylate groups from C^-H^+ are as shown in Table 2. [b] All cationic sites were assigned charges of $+1$, S_γ of Cys25 was assigned a charge of -1, and the C-terminus and the side chains of the Asp and Glu residues were initially assumed to be uncharged; ΔG was calculated as the energy required to place a charge of -1 on each carboxyl group in turn (a charge of -0.5 on each of the carboxyl O atoms).

Table 4: Electrostatic Energies of Interaction (ΔG) between the C^-H^+ Ion Pair of the Catalytic Site of Interleukin-1β Converting Enzyme (ICE) and Individual Carboxylate Anions within 20 Å in the Presence of All of the Positive Charges on the Protein

residue contributing the carboxylate anion[a]	$-\Delta G^b$ (kcal mol^{-1})	av distance[c] from the centroid of C^-H^+ (Å)
D174	13.0	16.4
D185	34.3	11.6
D210	5.0	19.7
D254	12.2	15.1
D288	3.3	8.3
D336	9.0	14.1
D381	5.4	17.9
E171	21.8	13.3
E172	15.5	15.3
E183	8.6	18.1
E241	3.5	16.0
E250	2.6	13.0
E390	31.8	15.8

[a] The prime candidate for the postulated electrostatic switch (D185) identified by the relatively large value of the interaction energy and its proximity in a somewhat hydrophobic environment (N184, M235, and I350) to the C285−H237 ion pair of the catalytic site and another potential candidate (E390) are underlined. [b] Values of ΔG were calculated as described in Table 3. [c] These distances are as defined in Table 2.

and caricain (see the Discussion section). Another potential candidate also based on proximity and interaction energy is Glu35. Analogous considerations suggest Asp185 to be the most likely candidate for an electrostatic switch in ICE that might perturb the Cys285−His237 catalytic site ion pair (see the Discussion section and Figure 6).

DISCUSSION

Use of Natural Structural Variants in the Papain Family of Cysteine Proteinases for Mechanistic Study. Studies on reaction mechanism require analysis of the kinetic consequences of structural variation in reactant state partners from which patterns of reactivity and their structural origins emerge. Enzymes such as proteinases that catalyze reactions of macromolecular substrates tolerate structural variation in the substrate or substrate-derived time-dependent inhibitor with retention of (perturbed) kinetic competence and thus allow the use of series of substrates and inhibitors in the construction of a structure−reactivity matrix. The particular value of the cysteine proteinase family derives from the natural variation within it of both structure and functional characteristics (Brocklehurst et al., 1987a; Brocklehurst, 1986) and from the existence of a highly reactive nucleophilic center ($-S^-$) in the common cysteine−histidine interactive system of the catalytic site which has a nucleophilic role in the catalytic act (Brocklehurst, 1987). The latter permits comparison of data from kinetics of catalysis with those from reactions with thiol-specific time-dependent inhibitors. These can be designed to involve single-step covalency change analogous to an individual step of the catalytic act. Suitably designed inhibitors are particularly effective as reactivity probes to assess the effects of both local molecular microenvironments and structural perturbations from ligand binding remote from the immediate catalytic site on the reactivity of the catalytically competent thiolate−imidazolium ion pair state of the enzyme as well as on that of the catalytically inactive uncomplicated thiolate anion (Brocklehurst et al., 1987b, 1988a). The considerable difference between the kinetic behavior of papain and that of some other cysteine proteinases suggests that study of other members of the family should contribute to establishing mechanistic concepts. Indications of such differences in the relatively little studied enzymes ficin (Malthouse & Brocklehurst, 1976; Shipton et al., 1976; Björk & Ylinenjärvi, 1990; Patel et al., 1993) and caricain (Topham et al., 1991; Taylor et al., 1994) identified these enzymes as prime candidates for detailed study to establish the number and characteristics of kinetically influential ionizations that determine catalytic site behavior. The status of the proton as the least sterically demanding general perturbant of protein structure and the importance of the protonation state of specific side chains in enzyme function make properly designed pH-dependent kinetic studies valuable in understanding enzyme mechanism and the chemical behavior of the active center (Brocklehurst, 1994, 1996b).

pK_a Values Characteristic of the Formation and Deprotonation of the Catalytic Site (Cys)-S^-/(His)-Im^+H (C^-H^+) Ion Pairs of Papain, Caricain, and Ficin. In order to investigate the relationship between the C^-H^+ ion pair content of a cysteine proteinase and catalytic competence, it is necessary to define the pH region in which each of these develops. A useful way to detect thiolate anion in both the ion pair (C^-H^+) and uncomplicated thiolate (C^-H) states is by use of a chromogenic, thiol-specific electrophilic probe reagent that reacts on the stopped-flow time scale to maximize the range of pH over which reactivity may be monitored without complication from denaturation. Interpretation is facilitated if the probe reagent does not change its ionization state over the pH range to be investigated. 4,4′-Dipyrimidyl disulfide (4PMDS; **1** ⇌ **2**; Scheme 1) is such a reagent. The low pK_a value of **1** (0.91) and consequent reaction essentially only with the unprotonated form **2** results in the simple double sigmoidal shapes of the pH−*k* profiles for the reactions of papain, caricain, and ficin with 4PMDS shown in Figure 1. The simplicity of these pH−*k* profiles and the requirement for $-S^-$ for nucleophilic character permit unambiguous assignments of the observed pK_a values. Thus as the pH is increased, generation of nucleophilic character occurs initially across pK_a values of 3.32 ± 0.01 in papain, 2.88 ± 0.02 in caricain, and 2.49 ± 0.02 in ficin. This increases subsequently across pK_a values 8.34 ± 0.04 in papain, 7.93 ± 0.05 in caricain, and 8.39 ± 0.02 in ficin. The initial generation of nucleophilic character is assigned to C^-H^+ formation and its subsequent enhancement to proton loss from the imidazolium component of the ion pair to provide the "uncomplicated" thiolate anion, C^-H. The marked variation in the pK_a values associated with C^-H^+ formation in the three enzymes is particularly noteworthy in connection with the major conclusion, here reported and discussed below, that generation of C^-H^+ does not provide catalytic competence.

The assignment of the pK_a values of 3.32 in papain and 2.88 in caricain to C^-H^+ formation is supported by the results of electrostatic potential calculations using finite difference solutions of the Poisson−Boltzmann equation. The lack of a 3D structure for ficin prevented calculation of the pK_a value of C^-H^+ formation in this enzyme. The results of these calculations reported in Table 1, in which the buried water molecules were retained and a protein dielectric constant of 2 was assumed, predict the pK_a value of C^-H^+ formation in caricain to be lower by 0.63 than that of the analogous value in papain. This value (2.69) is 93% of the value determined experimentally using 4PMDS (2.88), the pK_a shift from that of papain determined experimentally (−0.44) being 70% of the calculated value. Given that the techniques of calculating electrostatic effects are still being developed [e.g., by Wlodek et al. (1997) and Zhou and Vijayakumar (1997)], the correspondence between our calculated and experimental values of ΔpK_a can be considered to be good support for the assignment of these pK_a values to C^-H^+ formation. Even when the assumptions on which the calculations are based are varied as described below, the correspondence between calculated and experimental values of the pK_a for C^-H^+ formation in caricain is between 81% and 100%. In the absence of the buried water molecules there was a 10% change in the potentials generated at S_γ leading to a calculated value of $\Delta pK_a = -0.44$, identical with the value determined experimentally. We recently reported analogous calculations that accurately account for the pK_a difference between C^-H^+ ion pair formation in thiolsubtilisin Carlsberg and thiolsubtilisin BPN′ determined by reactivity probe kinetics (Plou et al., 1996). In that case good correspondence between the calculated and experimentally determined values of ΔpK_a (97%), assuming a protein dielectric constant of 2, required retention of the buried water molecules. The importance of bound water molecules for enzyme function is increasingly being recognized (Willenbrock et al., 1995). In the present work the effects of other structural perturbations on the calculated values of ΔpK_a serve to emphasize that as yet there is no established general protocol for calculating ΔpK_a values in all proteins. Although it seems possible that minimization of the protein structures at various stages of the modeling might be expected to contribute to the accuracy of the results (Warshel et al., 1986; Gilson &

Honig, 1988; Soman et al., 1989), the way in which this should be applied is not yet clear. Thus, when the structures of papain and caricain were subjected to small amounts of energy minimization with a 10.0 kcal mol^{-1} $Å^{-1}$ harmonic constraint on all non-hydrogen atoms but with no constraints on the side chains of Cys25 and His159, the change in the potentials at S_γ were of the order of 25% and the calculated value of ΔpK_a was −0.25 (only 57% of the experimental value). Although the structural perturbations caused by the minimizations involved maximum displacement among the atoms of only *ca.* 0.5 Å, clearly the cumulative effects on the multiple electrostatic environments can be quite substantial. At the present state of the art, it seems inadvisable to carry out energy minimization as part of the ΔpK_a calculation process, at least when comparing two different proteins. It has been necessary for a number of protein systems to assume an abnormally high protein dielectric constant of 20 to account for the observed pK_a shifts, acknowledging that this value takes account of various unknown factors not included explicitly in the model (Antosiewicz et al., 1994, 1996a). More recently, there have been reports of good agreement using more normal values of the protein dielectric constant [e.g, 4 (Antosiewicz et al., 1996b; Oberoi et al., 1996; Wlodek et al., 1997) and 2 in our own work on the thiolsubtilisins (Plou et al., 1996) and in the present work]. In the present work, when we changed the value of the protein dielectric constant from 2 to 20, the value of ΔpK_a was −1.0, which predicts a pK_a value for C^-H^+ formation in caricain of 2.32. In this case the experimentally determined value of ΔpK_a is only 68% of the calculated value.

The various factors that can contribute to error in electrostatic potential calculations were listed by Plou et al. (1996). The present work demonstrates, at least for papain and caricain, that a value of *ca.* 2 for the protein dielectric constant should be used and energy minimization should be omitted from the procedure. It is difficult to justify on physical grounds the omission of the buried water molecules even though in the present work this improves the calculated value of ΔpK_a from 70% to 100% of the experimental value. The accuracy of the results of the calculations can be accounted for in part by the use of finite element methods and focusing (Gilson, 1995; Honig & Nicholls, 1995) and the use of fast computers with large memories that permit the use of large grid sizes to survey initially a substantial region of space around the protein followed by subsequent implementation of finer grid resolutions (in the present work down to 0.1 Å, i.e., use of a final grid of 15 Å × 15 Å × 15 Å).

Possible Origins of the Variation in the Value of the pK_a for C^-H^+ Formation in Papain and Caricain. The results of an attempt to identify by calculation possible origins of the variation in this pK_a value (3.32 ± 0.02 for papain; 2.88 ± 0.02 in caricain) are shown in Table 2. None of the anionic groups makes a large contribution to the energy required to place a unit negative charge at S_γ of C^-H^+ uniquely to only one of the two enzymes. By contrast, the side chains of Lys64, Lys137, His18, and His61 in caricain, which are 10.7−13.4 Å from the catalytic site, each contribute *ca.* 0.5 kT to the shift in the pK_a for C^-H^+ formation. The residues at equivalent locations in papain all have noncationic side chains (Asn, Ala, Asn, and Tyr, respectively). The greater number of positive charges on caricain (36) than on papain (24) might contribute multiple electrostatic effects resulting in the lower pK_a values for C^-H^+ formation, but the calculations that produced the results in Table 2 suggest that the major effects arise from the four cationic side chains in caricain discussed above that are without analogue in papain.

The pH Dependence of k_{cat}/K_m for Substrate Hydrolysis Catalyzed by Papain, Caricain, and Ficin: Early Evidence of Multiple Catalytically Influential Ionizations. The parameter k_{cat}/K_m (Bender & Kézdy, 1965; Brocklehurst et al., 1968; Brot & Bender, 1969; Brocklehurst & Cornish-Bowden, 1976; Lowe, 1976; Brocklehurst, 1977; Fersht, 1977) is now widely used as an index of kinetic specificity and enzyme effectiveness. The pH dependence of this parameter and of the analogous second-order rate constant, k, for reactions with time-dependent inhibitors reveals free reactant state pK_a values (Brocklehurst & Dixon, 1976, 1977; Brocklehurst, 1979) when $k_{cat}/K_m << \sim 1 \times 10^7$ M^{-1} s^{-1}.

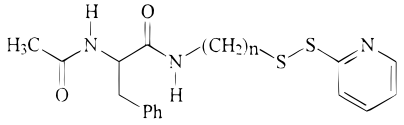
Early studies on the pH dependence of k_{cat}/K_m for the papain-catalyzed hydrolysis of a number of substrates suggested, often on the basis of scattered data over a relatively narrow pH range, two kinetically influential ionizations with pK_a values *ca.* 4 and 8.5 [see Brocklehurst et al. (1997)]. Reexamination of some of these pH dependences demonstrated improved fits to the data if a second ionization with pK_a 4 were included (Sluyterman & Widjenes, 1973; Lewis et al., 1978). This appeared to be in accord with the observation that the reactivity of the thiolate anion component of the (Cys25)/(His159) ion pair of papain is influenced by ionization with pK_a *ca.* 4 (Brocklehurst & Little, 1972). A particularly interesting collection of pH-dependent kinetic data for papain reactions in acidic media is that contained in the paper by Polgár and Halász (1978) which was subsequently reassessed by Mellor et al. (1993c). Polgár and Halász (1978) reported that although the rate of alkylation of papain by the simple alkylating agents, methyl and ethyl bromoacetate, depends upon a pK_a value of 3.25 and alkylation by chloroacetate on a pK_a value of 3.6, acylation by six electrically neutral substrates depends on a pK_a value of 4.0 and acylation by three cationic substrates (with Arg in P_1) on a pK_a value of 4.3. They analyzed each of their sets of pH-dependent kinetic data in terms of a single ionization curve and interpreted the marked variation that appears to occur in the kinetically influential pK_a value as the structure of the inhibitor or substrate is varied in terms of "fluctuation fit". This hypothesis allows substrates or inhibitors to select different conformations of the enzyme from an equilibrium stereo population and thereby, supposedly, provides for different pK_a values to be exhibited. This hypothesis did not survive analysis of a kinetic model of conformational selectivity, however, which demonstrated that the same pK_a value will always be obtained whether or not several rapidly fluctuant conformations react and whether or not different substrates or inhibitors react selectively with different conformations of the enzyme (Brocklehurst et al., 1983). An associated computer simulation study suggested a kinetically valid explanation of the interesting observations of Polgár and Halász (1978) in terms of ionizations with overlapping pK_a values.

The influence of multiple ionizations has been apparent not only from studies on papain but also from studies on other cysteine proteinases such as ficin and caricain. The pH dependence of ficin catalyzed hydrolysis of a number of substrates (mostly esters and amides of Bz-L-Arg, Cbz-L-Lys, and Bz-Gly) has been studied by several groups. The

pH−k_{cat}/K_m profiles (Whitaker & Bender, 1965; Hollaway et al., 1971) are approximately bell-shaped, maximal at pH *ca.* 6.5, and were analyzed in terms of two ionizations with pK_a values 4.5−5.0 and 8.5−8.7, respectively, despite the fact that inspection of the acid limb of these profiles always suggests the presence of at least two kinetically influential ionizations. The influence of multiple ionizations is suggested particularly clearly within the acid limb of the extensive pH−"activity" data set reported as Figure 1 by Hammond and Gutfreund (1959) despite the claim by these authors that the figure indicates how closely the experimental points fit to the line of a simple bell-shaped curve. Unfortunately, the data of Hammond and Gutfreund are not amenable to quantitative interpretation because the experimental design (use of initial rates and [S] $\sim$ K_m for most data points) is seriously flawed [see Brocklehurst (1994)]. The pH dependence of k_{cat}/K_m for caricain-catalyzed hydrolysis has revealed the existence of multiple ionizations (Topham et al., 1991; Taylor et al., 1994), but some of these data relate to ionic substrates.

The pH Dependence of k_{cat}/K_m for the Hydrolysis of N-Ac-Phe-Gly-4NA Catalyzed by Papain, Caricain, and Ficin: Evidence That the Catalytic Site Ion Pairs of These Enzymes Require Electrostatic Modulation To Become Catalytically Competent. The substrate chosen in the present work to evaluate catalysis, *N*-Ac-Phe-Gly-4NA, is the simplest chromogenic analogue substrate containing the major recognition features identified for papain, i.e., a hydrophobic side chain at the P_2 position and a P_1-P_2 amide bond which provides transcleft hydrogen bonding to the backbones of Gly66 and Asp158 (Brocklehurst et al., 1987a), and devoid of ionic substituents. The lack of ionic groups in the substrate was designed to reveal electrostatic effects within the enzyme molecule.

The data for the pH dependences of k_{cat}/K_m for the hydrolysis of this substrate catalyzed by the three cysteine proteinases are shown in Figure 2. Figure 2b,d,f shows theoretical pH−k_{cat}/K_m profiles for the hydrolysis of *N*-Ac-Phe-Gly-4NA catalyzed by papain, caricain, and ficin, respectively, constructed by assuming that catalytic ability is produced synchronously with and consequent upon (Cys)-S^-/(His)-Im^+H ion pair formation as the pH is increased across pK_a 3.32 for papain, 2.88 for caricain, and 2.49 for ficin. The concept that catalytic ability is produced by ion pair formation arose from early studies on the pH dependence of the kinetics of the acylation process, which appeared to produce conventional bell-shaped curves characterized by macroscopic pK_a values of *ca.* 4 and 8.5 [see, e.g., Smith and Parker (1958), Whitaker and Bender (1965), Lowe and Yuthavong (1971), and Ascenti et al. (1987) for studies on papain]. Studies on a model system involving reaction of 2-(mercaptomethyl)-*N*-methylimidazole with a distorted anilide demonstrated just such a form of pH dependence (Keillor & Brown, 1992). The fact that the evidence discussed below demonstrates the need for a different interpretation of the pH−k_{cat}/K_m profiles for the acylation processes in the enzyme-catalyzed reactions illustrates the limited value of studies on low M_r model systems. It is clear that lines b, d, and f in Figure 2 do not fit the data, particularly in acidic media. Thus, it is a striking observation that catalytic ability develops as the pH is increased across pK_a values *ca.* 4 (Figure 2a,c,e; pK_a 4.30 ± 0.04 for papain; pK_a 3.85 ± 0.03 for caricain; pK_a 4.03 ± 0.02 for ficin) rather than at lower pH values consequent upon C^-H^+ ion pair formation. Preliminary evaluation of the data in Figure 2 using SKETCHER suggested that catalytic activity might develop to a minor extent consequent upon C^-H^+ formation alone. Subsequent analysis by weighted nonlinear regression, however, produced the fits to the data shown in Figure 2a,c,e which are statistically indistinguishable from those for the more complex model containing the additional pK_a for C^-H^+ formation. Thus, there is no evidence that catalytic competence develops to any significant extent consequent upon C^-H^+ formation alone before the electrostatic switch triggered by deprotonation with pK_a *ca.* 4 has occurred. In summary, deprotonation with a pK_a value *ca.* 4 is kinetically essential in the catalysis of the hydrolysis of substrate **3** (Figure 2a,c,e), is kinetically dominant in the reaction of papain with substrate-derived 2-pyridyl disulfide probes **6**



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(Brocklehurst et al., 1988b; Patel et al., 1994) where rapid reaction at pH 6−7 occurs with the unprotonated probe by concerted attack of both components of the ion pair, and, as demonstrated below, is kinetically significant in the reactions of cysteine proteinases with the cationic probe **4** (Figure 3). It is the generation of the crucial electrostatic influence by deprotonation across pK_a *ca.* 4 that promotes catalytic competence and not, as has been generally supposed, formation of the key catalytic site motif (C^-H^+). A combination of this deprotonation and key binding interactions (Brocklehurst et al., 1987b, 1988b), as revealed by their ability to produce concerted attack by both components of the catalytic site ion pair of papain at the electrophilic center and leaving group of substrate derived probes **6** ($n = 1-3$), may control C^-H^+ ion pair geometry to permit each component to play its role in the chemistry of acyl enzyme intermediate formation.

An ionization with pK_a 10 provides minor but unmistakable modulation of the acylation process of the catalytic act (Figure 2). This deprotonation, which is probably remote from the active center region, has the effect of offsetting the decrease in k_{cat}/K_m resulting from loss of the C^-H^+ ion pair by increasing its intrinsic catalytic ability. Earlier indications that an ionization with pK_a *ca.* 10 might perturb the environment of the active center include those reported by Brubacher and Bender (1966), Hinkle and Kirsch (1970, 1971), and Lewis and Shafer (1974) for papain and by Topham et al. (1991) for both papain and caricain.

Reactivity Characteristics of the Catalytic Sites of Papain, Caricain, and Ficin Revealed by Reactions with the Two-Protonic-State Reactivity Probe, 2,2′-Dipyridyl Disulfide (2PDS): Comparison with the Characteristics Revealed by Reaction with 4PMDS and by Catalyzed Hydrolysis. The higher pK_a value (2.45) of the monocation of 2,2′-dipyridyl disulfide (**4**) compared with that (0.91) of the analogous pyrimidyl disulfide monocation (**1**) provides for significant reaction of the highly reactive cationic form of the probe **4** and the consequent striking rate maxima in acidic media for the reactions of all three enzymes. The existence of these rate maxima constitutes the most compelling evidence for nucleophilic character in the C^-H^+ ion pairs of the catalytic sites of these enzymes (Brocklehurst, 1996b). Also the

pH−*k* profiles in Figure 3 reveal the existence of kinetically influential ionizations with pK_a *ca.* 4 in the reactions of papain and ficin with the cationic probe **4** and suggest the possibility of an analogous ionization in caricain. It is noteworthy that this pK_a is a major characteristic of the pH dependence of k_{cat}/K_m for the hydrolysis of the substrate *N*-Ac-Phe-Gly-4NA (**3**) catalyzed by all three of these enzymes (Figure 2a,c,f). The discrepancy between the value of this common pK_a characteristic of the development of catalytic competence and the pK_a values characteristic of C^-H^+ ion pair formation in the three enzymes discussed above demonstrates an important new facet of cysteine proteinase mechanism.

The values of the characterizing parameters (pK_a values and values of pH-independent rate constants) of the pH dependence of k_{cat}/K_m for the hydrolysis of the 4-nitroanilide substrate **3** catalyzed by the three enzymes and of their reactions with 4PMDS and 2PDS are summarized in Table 5. The significance of many aspects of the shapes of the pH−*k* profiles and of their constituent pK_a values has been discussed above. The simplest profiles are those for the reactions with 4PMDS, where *k* increases with pH along two sigmoidal components. The reactive state characterized by $\tilde{k}_1$ involves reaction of the thiolate anion of the C^-H^+ ion pair with the neutral 4PMDS molecule **2**. In the more reactive state, characterized by $\tilde{k}_2$, the nucleophilicity of the thiolate anion would be expected to have increased consequent upon removal of the electrostatic influence of the imidazolium cation of the ion pair when C^-H^+ becomes C^-H. The more complex profiles for the reactions with 2PDS and its monocation, $2PDS^+H$, contain contributions from reactions of the C^-H^+ ion pairs both with ($\tilde{k}_2$) and without ($\tilde{k}_1$) enhancement of reactivity provided by the electrostatic switch (pK_a *ca.* 4) (at least with papain and ficin). It is noteworthy that the pK_a values associated with this switch in both papain (4.03 ± 0.05) and ficin (3.84 ± 0.02) are somewhat lower than the analogous values observed in catalysis (4.30 ± 0.04 for papain; 4.03 ± 0.02 for ficin). These differences in the pK_a values associated with the electrostatic switch could rise from the existence of overlapping ionizations in the switch mechanism. Thus if the reorganization of C^-H^+ geometry required for enhancement of reactivity of the thiolate anion component is somewhat different from that required for catalysis, it is possible that at least one additional ionizing group is involved in the switch mechanism for catalysis compared with that for enhancement of nucleophilicity. The switch pK_a values observed in catalysis (4.30 in papain and 4.03 in ficin) could each arise from two overlapping ionizations (4.03 and, say, *ca.* 5 in papain and 3.8 and, say, *ca.* 5 in ficin). Perturbation of pK_a values of apparently single ionization curves by overlapping ionizations has been demonstrated previously (Brocklehurst et al., 1983; Brocklehurst, 1994).

Possible Candidates for the Origin of the Kinetically Essential Electrostatic Influence Produced by Protonic Dissociation with pK_a ca. 4. Interest is growing in the contributions of electrostatic effects in the cysteine proteinase mechanism [see Duncan et al. (1992) for a discussion]. There has been particular interest over a long period in possible roles for the carboxylate side chains of Asp158 in papain and, more recently, in caricain [see Brocklehurst et al. (1987a) and Taylor et al. (1994) for discussions]. This is the closest charged residue to the catalytic site of the (Cys)-S^-/(His)-Im^+H ion pair and would seem to be the first choice candidate for the kinetically influential ionizations with pK_a *ca.* 4 in these enzymes discussed above. Evidence that this

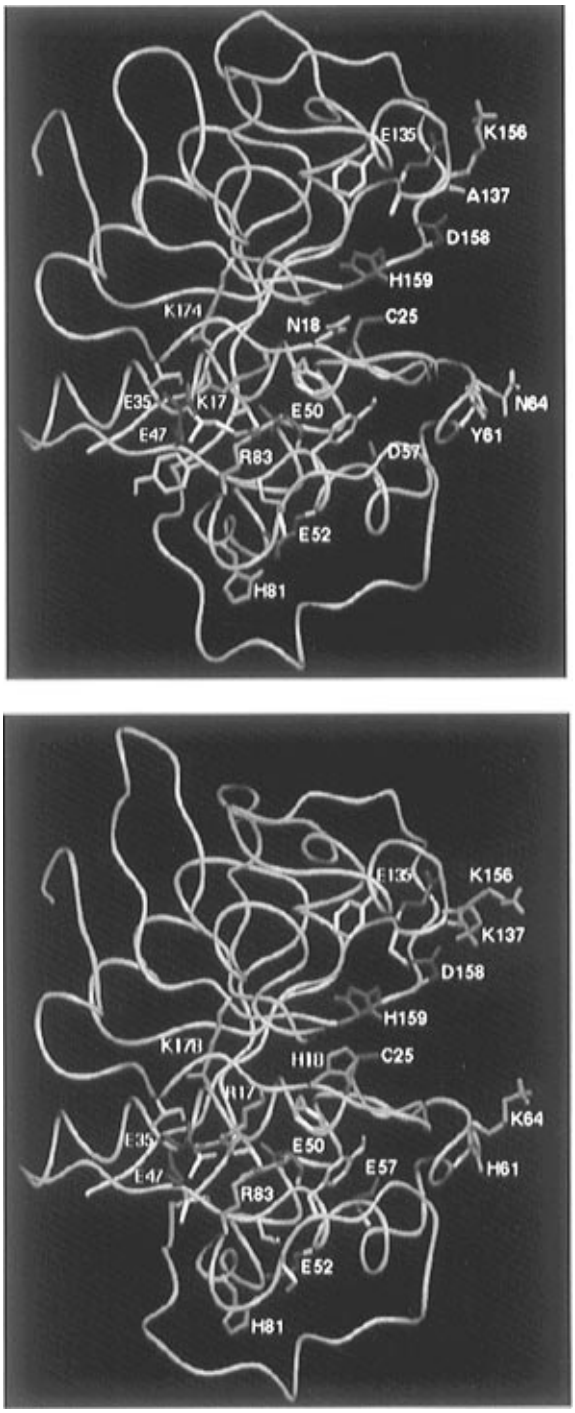

FIGURE 4: Views of (top) papain (from 9pap.pdb; Kamphuis et al., 1984) and (bottom) caricain (from 1ppo.pdb;Pickersgill et al., 1991) showing the catalytic site ion pair residues Cys25 and His159 (in magenta) and the two electrostatic clusters associated with Glu50 and Glu135, respectively (with Asp and Glu residues in red, Arg and Lys residues in blue, hydrophobic residues in white, nonionic polar residues in yellow, and the protein backbone in pale blue). Apart from Asp158, Glu50 and Glu135 contribute the nearest anionic side chains to the catalytic site C^-H^+ ion pairs. The distances between the centroids of the S_γ and $N_{\delta 1}$ of the ion pairs and the centroids of the oxygen atoms of the carboxylate groups of the specified residues are as follows: for papain, Glu50 12.94 Å, Glu135 12.91 Å; for caricain, Glu50 12.54 Å, Glu135 12.82 Å. The electrostatic clusters associated with Glu50 are closely similar in both enzymes, the only difference being that residue 17 is Lys in papain and Arg in caricain. By contrast, in the electrostatic clusters associated with Glu135 there is a significant difference, in that residue 137 is Ala in papain and Lys in caricain.

Table 5: Characterizing Parameters[a] of the pH Dependences of the Second-Order Rate Constants for the Reactions of the Catalytic Site Thiol Groups of Papain, Caricain, and Ficin with 4,4′-Dipyrimidyl Disulfide (4PMDS) and 2,2′-Dipyridyl Disulfide (2PDS) and of k_{cat}/K_m for the Hydrolysis of *N*-Ac-Phe-Gly-4NA Catalyzed by These Enzymes

	4PMDS reaction		catalysis			2PDS reaction			
enzyme	pK_1 (10^{-3} $\tilde{k}_1$)	pK_2 (10^{-5} $\tilde{k}_2$)	pK_1 (10^{-3} $\tilde{k}_1$)	pK_2 (10^{-2} $\tilde{k}_2$)	pK_3 ($\tilde{k}_3$)	pK_1 (10^{-4} $\tilde{k}_1$)	pK_2 (10^{-5} $\tilde{k}_2$)	pK_3 (10^{-2} $\tilde{k}_3$)	pK_4 (10^{-3} $\tilde{k}_4$)
papain	3.32	8.34	4.30	8.28	9.80[b]	2.40[c]	3.39	4.03	8.48
	(2.03)	(0.42)	(1.98)	2.18)	(0)	(0.06)	(0.32)	(5.48)	(1.54)
caricain	2.88	7.93	3.85	8.24	10.00[b]	2.44[d]	3.43[d]	7.88[d]	
	(1.96)	(1.73)	(0.40)	(0.32)	(0)	(2.74)[d]	(0.005)[d]	(20.0)[d]	
ficin	2.49	8.39	4.03	8.45	10.00[b]	2.17[c]	2.77	3.84	8.57
	(0.71)	(0.42)	(0.23)	(0.32)	(0)	(3.95)	(1.50)	(3.88)	(2.59)

[a] The characterizing parameters (pK_a values and pH-independent second-order rate constants, $\tilde{k}$, in M^{-1} s^{-1}, where for the catalyzed hydrolyses $\tilde{k} \equiv \tilde{k}_{cat}/\tilde{K}_m$) were determined by weighted nonlinear regression analysis, with weighting factors inversely proportional to k^2, of pH−*k* data collected at 25.0 °C and *I* 0.1 M; the standard errors on the parameters are given in the legends to Figures 1−3; in most cases those on the pK_a values are ≤ *ca.* 3% of the values and those on values of $\tilde{k}$ are ≤ *ca.* 10% of the values. [b] These pK_a values were fixed at values observed in reactions with substrate-derived reactivity probes such as 3-(acetamido)trimethylene 2-pyridyl disulfide [see Mannervik (1982) for a discussion of the value of combining regression analysis with additional information obtained independently]; their omission results in significantly worse fits to the data at high pH as assessed both by eye and by consideration of the mean sums of squares with an *F*-test. [c] These pK_a values relate to the pK_a value of $2PDS^+H$ (2.45) perturbed to different extents by its proximity to the pK_a values for formation of the C^-H^+ ion pair states in the three enzymes determined by reaction with 4PMDS (column 1) and calculated from, for papain, $K_1 = 10^{-2.45} + 10^{-3.32}$, and for ficin, $K_1 = 10^{-2.45} + 10^{-2.49}$; as in the case of p$K_a$ *ca.* 10, their omission results in significantly worse fits to the data at low pH. [d] These values relate to a three pK_a model in which pK_1 is the pK_a of $2PDS^+H$, pK_2 is suggested to be the resultant of overlap of the pK_a for C^-H^+ formation (2.88) and that of the electrostatic switch with pK_a *ca.* 4, and pK_3 (analogous to pK_4 for the reactions of the other enzymes with 2PDS) is the pK_a for conversion of C^-H^+ to C−H.

is not the case, however, is provided by the retention of this pK_a in the pH dependence of k_{cat}/K_m for the residual activity of Asp158Asn mutants of both papain (Ménard et al., 1990) and caricain (Taylor et al., 1994) and by the results of kinetic and spectroscopic studies using 4-chloro-7-nitrobenzofurazan (Nbf-Cl) as a reactivity probe and reporter group (Noble et al., 1997). These recent studies suggest pK_a values for the Asp158 carboxy groups of papain and caricain to be 2.8 and <2, respectively, substantially lower than the pK_a value *ca.* 4 for which a candidate is sought. Thus, it now seems unlikely that the carboxy group of Asp158 has a direct role in catalysis, although calculations suggest that it may contribute significantly to the electrostatic field to which the catalytic site in each enzyme is exposed [see Taylor et al. (1994)]. The lack of a central mechanistic role for Asp158 in papain and caricain is in accord with the fact that the analogous residues in cathepsins B and H are Gly(196) and Asn(165), respectively [see Willenbrock and Brocklehurst (1985) and Kowlessur et al. (1989a,b)]. A more peripheral electrostatic role in papain and caricain could be compensated for in some members of the family by contributions from other components of the electrostatic field around the active center regions.

With the most obvious candidate for the kinetically influential ionization with pK_a *ca.* 4 (Asp158) now ruled out, it is necessary to continue to search for the identity of this key side chain. Inspection of the crystal structures of papain and caricain reveals that the two electrostatic clusters nearest to the C^-H^+ ion pair in each enzyme are those containing Glu50 and Glu135, respectively (Figure 4). The latter includes Asp158. The distances between the centroids of the S_γ and $N_{\delta 1}$ of the ion pairs and the centroids of the oxygen atoms of the carboxylate groups of the specified residues are as follows: for papain, Glu50 12.94 Å, Glu135 12.91 Å; for caricain, Glu50 12.54 Å, Glu135 12.82 Å. The electrostatic clusters associated with Glu50 are closely similar in both enzymes, the only difference being that residue 17 is Lys in papain and Arg in caricain. This makes this cluster the favored candidate to contribute the ionization with pK_a *ca.* 4 essential for catalysis in both enzymes. By contrast, in the electrostatic clusters associated with Glu135 there is a significant difference, in that residue 137 is Ala in papain and Lys in caricain. This difference makes it unlikely that Glu135 contributes a common catalytically essential ionization with pK_a *ca.* 4. The possibility that Glu50 might contribute to the electrostatic switch with pK_a *ca.* 4 is supported by the results of the calculations reported in Table 3. Its relatively close proximity to C^-H^+ (12.9 Å in papain and 12.5 Å in caricain) and the relatively large values of the interaction energies (−37.5 kcal mol^{-1} for papain and −52.7 kcal mol^{-1} for caricain) suggested Glu50 as the prime candidate for at least one component of the electrostatic switch. The larger value of the interaction energy for caricain is consistent with the lower value for the pK_a of the electrostatic switch in this enzyme revealed by kinetics of catalysis (3.85 ± 0.03) compared with that of papain (4.30 ± 0.04, Table 5). A lower value for the pK_a of Glu50 in caricain than that in papain might be predicted by the observation of an additional hydrogen bond donated to this side chain in the former enzyme (Figure 5).

The electrostatic influences required to endow the C^-H^+ ion pairs with catalytic competence appear to be linked, at least in some cases, with key P_2-S_2 binding interactions. Less than optimal P_2-S_2 hydrophobic contacts or cationic substituents in the substrate or probe appear to require additional protonic dissociations for full expression of catalytic ability (Brocklehurst et al., 1987a; Topham et al., 1991). These also might derive from one or other of the electrostatic clusters near the active center. The precise mechanism by which the electrostatic and binding effects provide for catalytic competence in the preexisting C^-H^+ ion pair remains to be discovered. It seems possible, however, that there may be a requirement to rearrange the ion pair geometry from that of an intimate ion pair to one in which the two components have separated to permit each to play its role in the rate-determining acylation process of the catalytic act, i.e., the thiolate anion as the attacking nucleophile and the imidazolium cation as the general acid catalyst of leaving group departure. Evidence that specific binding interactions (the hydrophobic P_2-S_2 effect and the hydrogen bonding of the P_1-P_2 amide bond across the active center cleft) provide rearrangement of transition state geometry in reactions with 2-pyridyl disulfide probes of type **6** such that the attack of the enzyme thiolate anion is concerted with

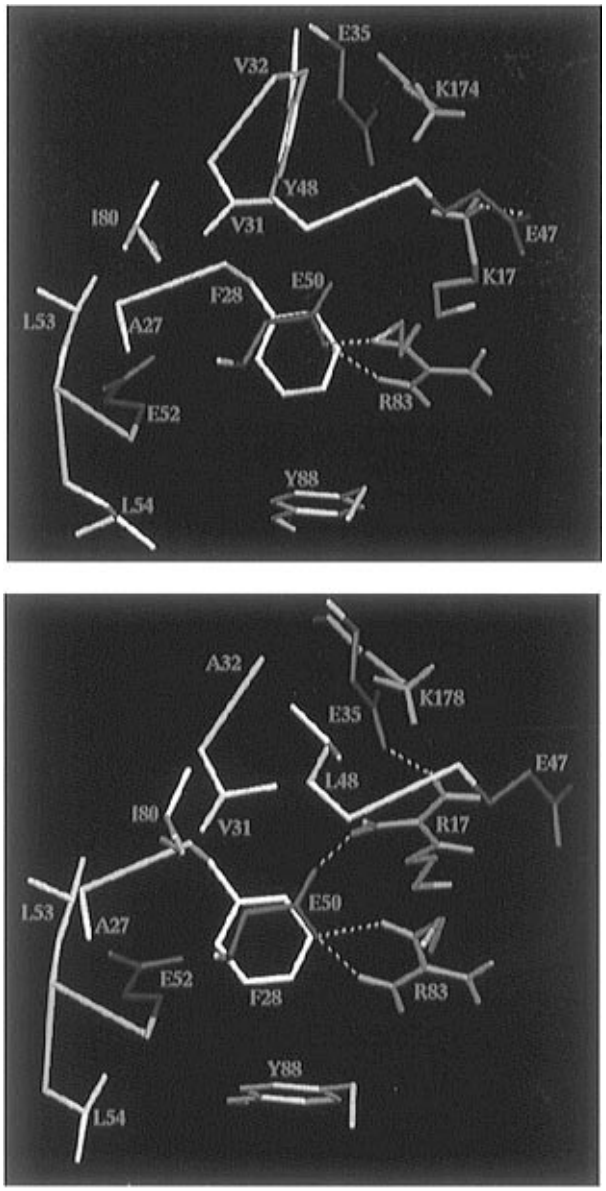

FIGURE 5: Views of the electrostatic clusters associated with Glu50 (a, top) in papain and (b, bottom) in caricain (color coded as in Figure 4). In each case the carboxy group of Glu50 is in a hydrophobic environment (the distances of closest approach to Phe28 being 3.5 Å in papain and 3.6 Å in caricain and to Val31 being 4.0 Å in papain and 3.6 Å in caricain) and is engaged in electrostatic-cum-hydrogen-bonding interaction with Arg83. A consequence of the difference in residue 17 (Lys in papain and Arg in caricain) is an additional hydrogen bond to the carboxylate of Glu50 in caricain which is not present in papain. This might contribute to the somewhat lower value of the pK_a suggested to be characteristic of Glu50 in caricain (3.85 ± 0.03) than in papain (4.30 ± 0.04) (see Table 5).

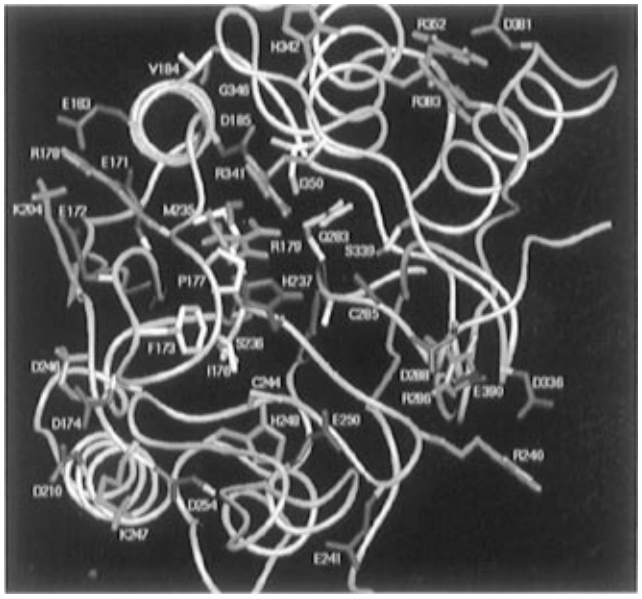

FIGURE 6: View of the active center region of ICE and the electrostatic field within 20 Å of the Cys285−His237 catalytic site ion pair (from 1ice.pdb; Wilson et al., 1994; color coded as in Figure 4). Asp185, suggested to be the electrostatic switch functionally analogous to Glu50 in papain and caricain, is located in a partially buried environment and is 11.6 Å from the centroid of the catalytic site ion pair with a relatively high interaction energy ($\Delta G = -34.3$ kcal mol^{-1}; Table 4).

interaction of the imidazolium cation with the pyridyl N atom of the leaving group has been reported (Kowlessur et al., 1989a; Patel et al., 1992).

Does the Concept of an Electrostatic Switch Apply to Other Members of the Cysteine Proteinase Superfamily? To begin to examine this question, we carried out an investigation of ICE by comparison of the catalytic site structure and the electrostatic characteristics within 20 Å of the C^-H^+ ion pair with those of papain. Wilson et al. (1994) determined the crystal structure of ICE at 2.6 Å resolution and pointed out its novel characteristics within the superfamily in terms of global fold, topology, and quaternary structure. The catalytic site C^-H^+ ion pair of ICE and the structure within 20 Å of this key motif are shown in Figure 6. Whereas in papain the catalytic site cysteine residue (Cys25) is located at the N-terminus of an α-helix, the functionally analogous residue in ICE (Cys285) is at the C-terminal end of a *β*-strand. The catalytic site region of ICE is substantially different from that of papain in a number of respects. In papain, the other component of the ion pair, His159, is shielded on one side by Lys134, Ala136, Ala160, Val161, and, notably, Trp177 [see, e.g., Brocklehurst et al. (1997)] and by Trp19, Asn175, and Asn176 on the other. In ICE, however, the catalytic site His237 is relatively exposed and is shielded mainly only on one side, by Phe173, Ile176, and Pro177. In papain Asn175 is hydrogen bonded to His159 as a component of the catalytic triad. In ICE, although Ser236 occupies a position somewhat similar to Asn175 in papain, the orientation of His237 is very different from that of His159, and a comparison between the two enzymes in this respect is difficult. The roles of Ile176 and Pro177 in ICE are analogous to those of Ala136 and Trp177 in papain, respectively.

As was pointed out by Wilson et al. (1994) ICE does not contain a carboxylate analogous to that of Asp158 in papain in that the residue adjacent to the catalytic site His237 on the N-terminal side is Ser236. In terms of electrostatic stabilization of the C^-H^+ ion pair, however, Asp288, on the Cys285 side of C^-H^+, provides stabilization by 3.3 kcal mol^{-1} which is comparable to that (2.6 kcal mol^{-1}) provided by Asp158 in papain, which is on the His159 side of C^-H^+. The entrance to the active center cleft in ICE is lined by several positively and negatively charged residues, and the electrostatic field is strongly polarized. A major difference between ICE and papain is that there are two cationic residues (Arg179 and Arg341) close to the C^-H^+ ion pair in the former.

The pH dependence of ICE catalytic activity (k_{cat}/K_m) was reported by Wilson et al. (1994) to be "a sharp bell-shaped curve with a maximum at pH 7.5, with component pK_as of

5.5 and 8.2" (data not shown). The pK_a of 8.2 is similar to the values of pK_2 for the pH dependence of k_{cat}/K_m (Figure 2 and Table 5) for catalyses by papain (8.28 ± 0.05), caricain (8.24 ± 0.06), and ficin (8.45 ± 0.04) and could well characterize proton loss from the catalytically active C^-H^+ state. If this is the case, it seems unlikely that the pK_a value of 5.5 characterizes the formation of the C^-H^+ state in ICE as the pK_a values for the analogous process are very much lower in papain (3.32 ± 0.01), caricain (2.88 ± 0.02), and ficin (2.49 ± 0.02) (Figure 1 and Table 5). It seems more likely that the pK_a 5.5 characterizes an electrostatic switch in ICE analogous to those with pK_a *ca.* 4 identified in the present work for papain, caricain, and ficin.

Examination of the partial structure of ICE (Figure 6) and the results of the electrostatic calculations (Table 4) analogous to those carried out for papain and caricain (Table 3) suggest Asp185 as the most likely candidate for the putative electrostatic switch. Its relative proximity to the C^-H^+ ion pair (11.6 Å), the relatively large interaction energy ($\Delta G = -34.3$ kcal mol^{-1}), and its location in a partially buried environment combine to support the tentative assignment of the carboxy group of Asp185 as the kinetically influential ionization with pK_a 5.5 in ICE.

Concluding Comment: A New Perception of the Acylation Process in the Cysteine Proteinase Mechanism. The results here reported demonstrate that an electrostatic switch is required to permit the thiolate and imidazolium components of the preformed catalytic site C^-H^+ ion pair to provide catalysis. When the substrate is nonionic and contains Phe as the occupant of the hydrophobic S_2 subsite and Gly in the P_1 position, the switch in papain consists of a single protonic dissociation with pK_a *ca.* 4 which cogent argument postulates might be assigned to Glu50. The analogous residue in caricain provides one component of a similar electrostatic switch. The key electrostatic switch in papain is postulated to combine with signaling effects from specific binding in the S_2 subsite and S_1-S_2 intersubsite regions (Kowlessur et al., 1989a; Patel et al., 1992) to provide the appropriate C^-H^+ ion pair geometry necessary to permit nucleophilic attack by the thiolate anion and general acid catalyzed assistance to leaving group departure by the imidazolium cation. With ionic substrates and those in which interactions in the various subsites vary in quality, additional contributions from binding and other electrostatic effects appear to refine the reactivity characteristics of the ion pair particularly in caricain (Topham et al., 1991; Taylor et al., 1994; Wang et al., 1994; Brömme et al., 1996). Modeling suggests that Asp185 might provide an analogous electrostatic switch in ICE. It may be, therefore, that the concept of essential modulation of the catalytic site C^-H^+ ion pair might apply generally within the cysteine proteinase superfamily. The realization that modulation of the C^-H^+ ion pair of this class of enzyme is necessary for catalytic competence and that in many cases the pK_a value thought to characterize C^-H^+ formation has been wrongly assigned (Ménard et al., 1991; Taylor et al., 1994; Wang et al., 1994; Ménard et al., 1995; Vernet et al., 1995; Brömme et al., 1996; Katerelos & Goodenough, 1996) requires reinterpretation of much of the data reported previously on the pH dependence of k_{cat}/K_m for catalysis by both native and mutated cysteine proteinases.

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