Peptide Aldehydes and Nitriles as Transition State Analog Inhibitors of Cysteine Proteases†

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ABSTRACT: Enzymes efficiently catalyze reactions by stabilizing inherently unstable transition states. For cysteine proteases, part of the stabilization is provided by a region of the enzyme termed the oxyanion hole. Site-directed mutagenesis has been used to investigate further the role of the oxyanion hole of papain in the binding of putative transition state analog inhibitors of cysteine proteases. The dissociation constants K_i^{obs} for inhibition of wild-type and mutant enzymes (Gln19Ala, Gln19Glu, and Gln19His) by the aldehyde Ac-Phe-Gly-CHO and the nitrile MeOCO-Phe-Gly-CN have been determined in the pH range 3.5−9.0. For the peptide nitrile inhibitor, mutation of Gln19 was found to cause important increases in K_i^{obs}, and thioimidate adducts with the papain mutants Gln19Ala and Gln19Glu are less stable by 1.4−2.4 kcal/mol. However, for the peptide aldehyde inhibitor, the mutations resulted in a small but significant increase in stability of the tetrahedral hemithioacetal adduct (0.4−1.2 kcal/mol). In that respect, the hemithioacetal formed between papain and a peptide aldehyde cannot be considered a good model of the transition state for cysteine protease-catalyzed reactions. The influence of the mutations on the pH dependency of inhibition also indicates that with respect to oxyanion hole interaction, the inhibition of papain by peptide nitriles is a process closer to that of substrate hydrolysis than is the inhibition by the corresponding peptide aldehydes. The nature of the intermediates and transition states in hydrolysis reactions catalyzed by cysteine proteases, as well as the use of enzyme−inhibitor adducts as their models, is discussed.

The overall mechanism of substrate hydrolysis by cysteine proteases is considered to consist of a number of steps involving the formation of a covalent acyl-enzyme and of transient anionic tetrahedral intermediates and transition states (Lowe, 1970; Glazer & Smith, 1971; Storer & Ménard, 1994). Most often cited as experimental support for the formation of tetrahedral intermediates is the potent inhibition of these enzymes by peptide aldehydes. These inhibitors form covalent tetrahedral adducts, hemithioacetals, with the active site cysteine residue (Bendall et al., 1977; Lewis & Wolfenden, 1977b; Mackenzie et al., 1986; Schröder et al., 1993). It has been demonstrated that peptide aldehydes are powerful reversible inhibitors of cysteine proteases (Aoyagi et al., 1969; Westerik & Wolfenden, 1972), and the potency of this inhibition is suggested to be due to the covalent adducts mimicking the tetrahedral transition states on the enzyme reaction pathway (Bendall et al., 1977; Frankfater & Kuppy, 1981).

Peptide nitriles are also good reversible inhibitors of cysteine proteases (Lucas & Williams, 1969; Sluyterman & Wijdenes, 1973; Lewis & Wolfenden, 1977b). The inhibitor reacts at the active site cysteine to form a covalent thioimidate bond with the thiol group, as shown by NMR experiments (Brisson et al., 1986; Moon et al., 1986; Liang & Abeles, 1987). This complex could conceivably undergo hydrolysis to an amide, and this has been observed, but only in the presence of very high concentrations of papain (Gour-Salin et al., 1991). Hydrolysis of the nitrile is therefore extremely slow, which explains why it has been considered to be nonexistent (Sluyterman & Wijdenes, 1973; Lewis & Wolfenden, 1977b; Liang & Abeles, 1987). The carbon atom of the thioimidate has an sp^2 hybridization and adopts a trigonal geometry. The geometry of the adduct is therefore different from that of the tetrahedral hemithioacetals formed between cysteine proteases and aldehydes. The structure of the complex formed with a peptide nitrile therefore resembles more closely the acyl-enzyme intermediate than the tetrahedral transition state in the catalytic pathway for substrate hydrolysis (see Figure 1). However, experimental data suggest that peptide nitriles behave as transition state analog inhibitors. Correlations were found between K_i and k_{cat}/K_M for peptide nitriles and the corresponding substrates, suggesting that the papain−nitrile complex has similarities with the transition state for substrate hydrolysis (Liang & Abeles, 1987; Hanzlik et al., 1990). In addition, peptide nitriles were found to interact with cysteine proteases in essentially the same way as do the hemithioacetals, i.e., a linear free energy correlation of 0.99 was found for the binding of 11 pairs of peptidyl aldehydes and nitriles (Hanzlik et al., 1990, 1991). The finding that aldehyde and nitrile peptides might behave as transition state analogs for papain is particularly striking in view of the differences in geometry and hybridization at the reactive carbon center in thioimidates and hemithioacetals. This raises questions regarding the exclusively tetrahedral nature of the transition state(s) on the cysteine protease reaction pathway.

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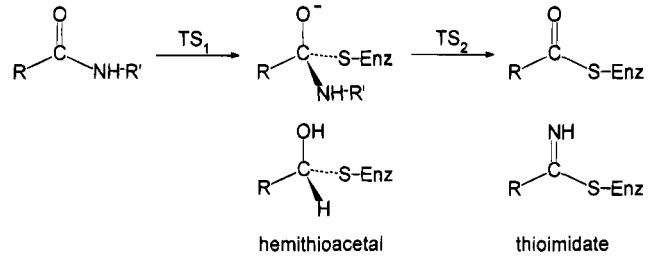


FIGURE 1: Comparison of the geometries of hemithioacetal and thioimidate adducts of papain with intermediates in the reaction pathway for substrate hydrolysis. TS_1 and TS_2, transition states in the reaction pathway.

Enzymes are able to efficiently catalyze substrate hydrolysis by stabilizing inherently unstable transition states. It has been shown that for cysteine proteases, part of the stabilization is provided by a region of the enzyme termed the oxyanion hole (Ménard et al., 1991a, 1995). This region in papain is defined by two hydrogen bond donors located on the backbone amide nitrogen of Cys25 and the side chain amide nitrogen of Gln19 (Drenth et al., 1976). The contribution of the oxyanion hole to transition state stabilization has been demonstrated using site-directed mutagenesis. Mutation of Gln19 to alanine and serine caused a 60- and 600-fold decrease in k_{cat}/K_M, respectively (Ménard et al., 1991a). More recently, it was shown that ionization of a glutamic acid residue introduced at position 19 in papain by site-directed mutagenesis modulates the pH−activity profile of the enzyme, providing additional support for the interaction between a substrate and the side chain of residue 19 in the oxyanion hole (Ménard et al., 1995). These results clearly indicate that the oxyanion hole constitutes part of the catalytic machinery of cysteine proteases. Considering that peptide aldehydes form hemithioacetals that are postulated to represent good models of tetrahedral transition states, the binding of these inhibitors to papain should also be strengthened by interactions in the oxyanion hole of the enzyme. However, mutation of Gln19 to Ala and Ser in papain was found to result not in a decrease but in a slight increase in affinity of the enzyme for the aldehyde-based inhibitor (Ménard et al., 1991a). It has been suggested by Mackenzie et al. (1986), on the basis of modeling, that the hydroxyl group of the tetrahedral hemithioacetal is not located in the oxyanion hole of papain. This is in disagreement, however, with the X-ray crystallographic structure of a papain−leupeptin complex (Schröder et al., 1993). In the structure of this complex, the inhibitor forms a hemithioacetal with Cys25 with the oxygen atom sitting within the enzyme's oxyanion hole, an observation supportive of the formation and stabilization of tetrahedral species on the catalytic pathway of cysteine proteases.

We have used site-directed mutagenesis to investigate further the role of the oxyanion hole in the binding of putative transition state analog inhibitors of cysteine proteases. Three mutants of papain (Gln19Ala, Gln19Glu, and Gln19His) have been expressed in the baculovirus−insect cell system (Vernet et al., 1990). The secreted precursors were converted *in vitro* to mature enzymes and purified to homogeneity, and the kinetic parameters for inhibition of the mutant enzymes by the aldehyde Ac-Phe-Gly-CHO[1] and the nitrile MeOCO-Phe-Gly-CN have been determined in the pH range 3.5−9.0. The mutants Gln19Glu and Gln19His were chosen because the charge state of the side chain introduced at position 19 could change within the pH range where the enzyme is normally active. It has been shown previously that ionization of the Glu19 side chain in Gln19Glu modulates the activity of the enzyme against substrates, allowing a clear visualization of the interaction between the substrate and residue 19 from pH−activity profiles (Ménard et al., 1995). The intrinsic reactivity of the carbon center in peptide aldehydes and nitriles against small thiol compounds has also been studied to establish a basis for comparison of the relative potencies of these two classes of inhibitors.

MATERIALS AND METHODS

The substrate CBZ-Phe-Arg-MCA was purchased from IAF Biochem International Inc. (Laval, Québec). Acetylphenylalanylglycinaldehyde diethyl acetal was a generous gift from Dr. M. Pozsgay (Institute for Biological Sciences, National Research Council of Canada). This compound was conveniently hydrolyzed to the corresponding peptide aldehyde, Ac-Phe-Gly-CHO, by incubation in 0.01 M HCl solution. The peptide nitrile MeOCO-Phe-Gly-CN was synthesized as described previously (Lowe & Yuthavong, 1971; Storer et al., 1982). Small thiol compounds (2-ME, 3-MPA) were purchased from Aldrich. Papain was obtained as a crystallized suspension in sodium acetate from Sigma Chemical Co. and was further purified, activated, and the active site titrated as described previously (Ménard et al., 1990). The mutants Gln19Ala, Gln19Glu, and Gln19His were prepared as described previously (Ménard et al., 1991a, 1995). The mutants of papain were expressed in insect cells infected with recombinant baculoviruses (Vernet et al., 1990). The secreted papain precursors were activated *in vitro* and then purified (Ménard et al., 1991b).

Kinetic Measurements. All kinetic measurements were performed at 25 °C as previously described (Ménard et al., 1995). The substrate CBZ-Phe-Arg-MCA was used to monitor enzyme activity. The concentration of recombinant enzymes was determined by measuring the initial rate for hydrolysis of CBZ-Phe-Arg-MCA at various substrate concentrations well below K_M and using the reported k_{cat}/K_M value of the enzyme to calculate the active enzyme concentration. The dissociation constants (K_i^{obs})[2] for inhibition of wild-type and mutant papain by Ac-Phe-Gly-CHO and MeOCO-Phe-Gly-CN were determined at pH between 3.5 and 9.0 by measuring the rate of substrate hydrolysis (v_s) in the presence of varying concentrations of inhibitor (0.2−5 × K_i^{obs}) and at substrate concentration kept well below K_M. Inhibitor and substrate concentrations were kept in excess over enzyme concentration. The K_i^{obs} value at a given pH

[1] Abbreviations: CBZ-Phe-Arg-MCA, carbobenzoxy-L-phenylalanyl-L-arginine methylcoumarinyl-7-amide hydrochloride; Ac-Phe-Gly-CHO, acetyl-L-phenylalanyl-glycinaldehyde; MeOCO-Phe-Gly-CN, methoxycarbonyl-L-phenylalanyl-aminoacetonitrile; 2-ME, 2-mercaptoethanol; 3-MPA, 3-mercaptopropionic acid; Glu^0, Glu^-, glutamic acid residue with side chain in the neutral and ionized states, respectively.

[2] The following convention is used to describe kinetic data: K_i^{obs} represents the dissociation constant of an enzyme−inhibitor complex determined at a given pH; K_i^{app} represents the same dissociation constant but in the presence of an added thiol compound which can react with the inhibitor; K_i is the limiting (pH-independent) value of the dissociation constant obtained by nonlinear regression of pH−K_i^{obs} data to a specified equation, while pK_j represent the corresponding experimental pK_a values. The data for wild-type enzyme refer to the commercially available wild-type papain obtained from papaya.

was obtained from a graph of $1/v_s$ vs [inhibitor] (Dixon, 1953). Under certain conditions with Ac-Phe-Gly-CHO, 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 product vs time progress curves were also used to obtain the kinetic parameters k_1 and k_2 of Scheme 1, which represents the simplest model describing a slow inhibition process (Cha, 1975), by fitting the progress curves at different inhibitor concentrations to eqs 1 and 2 (Cha, 1975; Williams & Morrison, 1979).

Scheme 1

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI$$

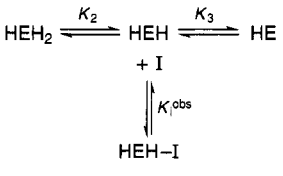
$$[P] = v_s t + \frac{(v_0 - v_s)[1 - \exp(-k_{obs}t)]}{k_{obs}} \quad (1)$$

$$k_{obs} = k_1[I] + k_2 \quad (2)$$

where v_s is the final steady-state rate, v_0 is the initial rate, and k_{obs} is the apparent first-order rate constant to reach steady state. The inhibition of wild-type papain by Ac-Phe-Gly-CHO was rapid, and dilution experiments were used to determine k_1 and k_2. Papain−inhibitor complexes were formed at various inhibitor concentrations between 0.2 and 1.0 μM. After 1 h incubation, the papain−inhibitor complex was diluted 100-fold with phosphate buffer, pH 7, containing the substrate (20 μM), and the progress curves to reach the new steady state were monitored.

The pH dependency of inhibition is reported in plots of $1/K_i^{obs}$ vs pH. For substrate hydrolysis, the pH dependency of activity can be described by a model in which three ionizable groups modulate activity of the enzyme (Ménard et al., 1990). The precision of the data in plots of $1/K_i^{obs}$ vs pH is not sufficient to differentiate between one or two groups affecting the activity in the acid limb, and therefore, pH−inhibition profiles have been analyzed by a minimum model, considering only two ionizable groups (Scheme 2), and the data were fitted to the corresponding equation (eq 3).

Scheme 2

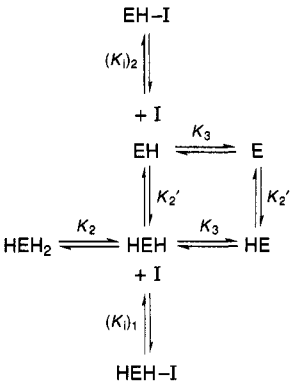


$$\frac{1}{K_i^{obs}} = \frac{1/K_i}{[H^+]/K_2 + 1 + K_3/[H^+]} \quad (3)$$

For the Gln19Glu mutant, the pH-inhibition profile with MeOCO-Phe-Gly-CN is best described by Scheme 3, and the data were fitted to eq 4.

In the models represented in Schemes 2 and 3, the numbering system used to describe the pK_a values is the same as the one used by Ménard et al. (1995), to facilitate comparison with previous studies. As will be shown later, the single pK_a determined in the acid limb is in agreement with the values of pK_2 reported previously.

Scheme 3



$$\frac{1}{K_i^{obs}} = \frac{1/(K_i)_1}{[H^+]/K_2 + (1 + K_3/[H^+])(1 + K_2'/[H^+])} + \frac{1/(K_i)_2}{[H^+]^2/K_2K_2' + (1 + K_3/[H^+])(1 + [H^+]/K_2')} \quad (4)$$

NMR Experiments. ^{13}C NMR samples were prepared in 25 mM borate buffer, pH 9.0, containing 0.5 mM EDTA, 5% D_2O, and 5% acetone. All spectra were recorded at 298 K using a Bruker AM 500 spectrometer operating at 125 MHz. The ^{13}C spectra were proton decoupled and were collected with 32K data points over a range of 30 kHz (260 ppm), using a pulse width of 4.2 μs and a relaxation delay of 1 s. The methyl carbon resonance of acetone assigned at 30.7 ppm was used as an internal standard. Concentration of MeOCO-Phe-Gly-^{13}CN was kept at 3 mM.

RESULTS

The dissociation constants, K_i^{obs}, for the complexes formed between papain and the peptide inhibitors have been measured in the pH range 3.5−9.0. The results are illustrated in Figure 2 for inhibition of wild-type and mutant enzymes by Ac-Phe-Gly-CHO and MeOCO-Phe-Gly-CN. The data are reported in graphs of $1/K_i^{obs}$ (i.e., the association constant) vs pH, since in this form the pH dependency curves reflect ionization of residues on the free enzyme that have an effect on the inhibition (Fersht, 1985). For wild-type papain, it can be seen that for both inhibitors, the pH dependency curve is bell-shaped and can be described by a minimum model (Scheme 2), in which two residues modulate the affinity of the enzyme for the inhibitor. The experimentally determined pK_a values describing the profile are reported in Table 1, along with the corresponding pK_a values derived from pH−activity curves for substrate hydrolysis (Ménard et al., 1991a, 1995). Considering the precision of the pH−inhibitory activity profiles, it can be seen that the same groups that control enzymatic activity against substrates are essential for binding of the inhibitors, indicating that the thiolate−imidazolium form of the enzyme is required for reaction with both the nitrile and the aldehyde derivatives. The limiting dissociation constants, K_i, obtained from these pH dependency curves are reported in Table 1.

For the peptide nitrile inhibitor, mutation of Gln19 to Ala, Glu, or His affects the pH−inhibitory activity profile in a manner very similar to what is observed for substrates. For all three mutants, the pK_a values reported in Table 1 are shifted relative to wild-type enzyme but are identical, within experimental error, to those obtained for substrate hydrolysis by the same mutants (Ménard et al., 1991a, 1995). The

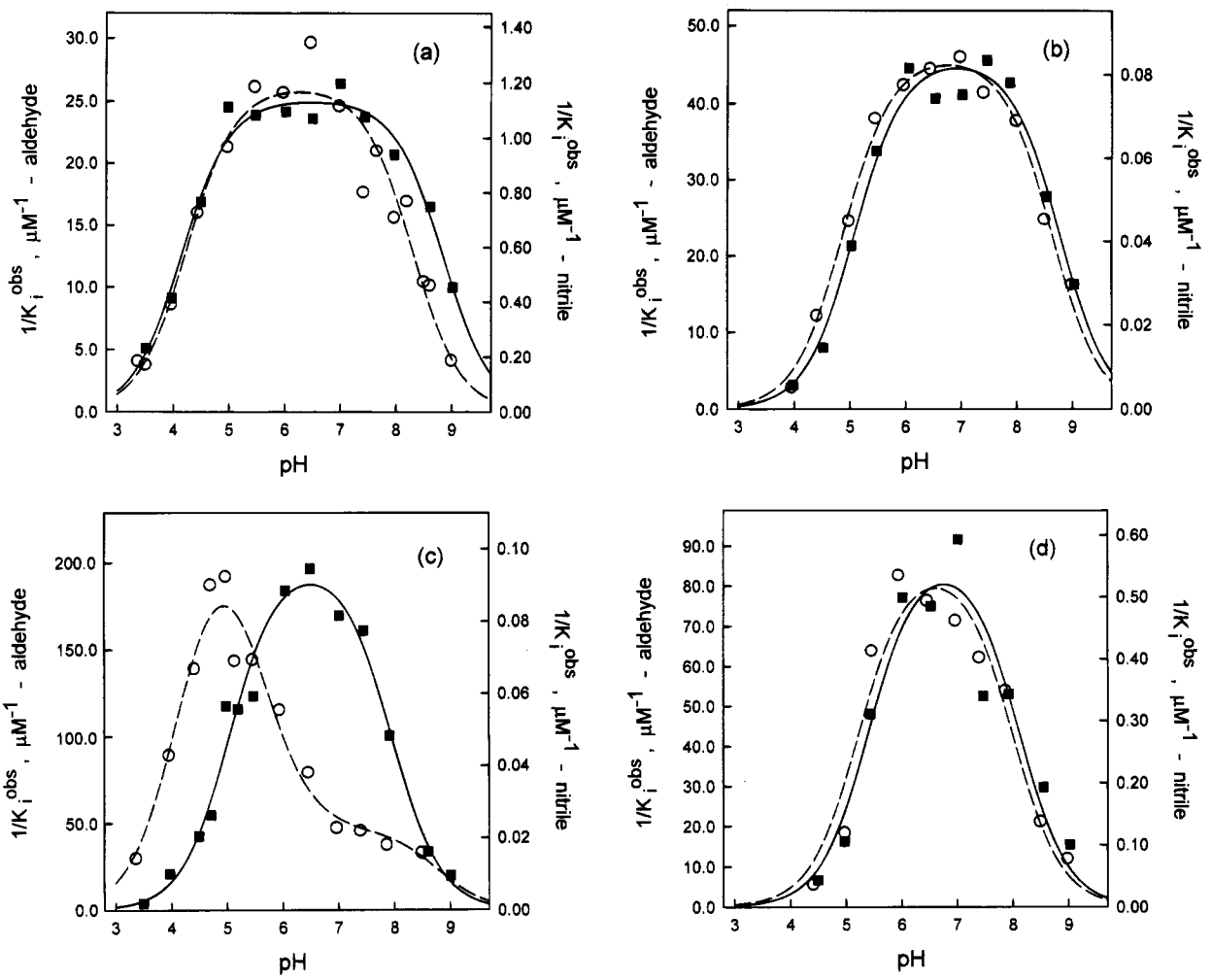


FIGURE 2: pH dependence of $1/K_i^{obs}$ for inhibition of (a) wild-type, (b) Gln19Ala, (c) Gln19Glu, and (d) Gln19His papain by Ac-Phe-Gly-CHO (■) and MeOCO-Phe-Gly-CN (○). The lines represent the best fit of the data to the models described in the text (solid lines for the aldehyde, and dashed lines for the nitrile).

Table 1: Analysis of pH−Inhibition Profiles with Ac-Phe-Gly-CHO and MeOCO-Phe-Gly-CN and of pH-Activity Profiles with CBZ-Phe-Arg-MCA for Mutant and Wild-Type Papain

enzyme	compound	K_i (μM)[a]	pK_1	pK_2	pK_2'	pK_3
papain	nitrile	0.84 ± 0.03		4.24 ± 0.11		8.28 ± 0.09
	aldehyde	0.0398 ± 0.0009		4.14 ± 0.07		8.84 ± 0.07
	substrate[b]		3.58 ± 0.29	4.54 ± 0.29		8.45 ± 0.02
Gln19Ala	nitrile	11.9 ± 0.2		4.88 ± 0.05		8.62 ± 0.05
	aldehyde	0.0218 ± 0.0006		5.08 ± 0.07		8.76 ± 0.08
	substrate[b]		4.1 ± 0.2	5.02 ± 0.04		8.51 ± 0.05
Gln19Glu	nitrile	9.1 ± 1.1 (Glu^0) 45 ± 12 (Glu^-)		4.13 ± 0.16	5.63 ± 0.23	8.79 ± 0.83
	aldehyde	0.0050 ± 0.0002		5.04 ± 0.07		7.95 ± 0.09
	substrate[b]		3.76 ± 0.27	4.37 ± 0.13	6.02 ± 0.06	8.86 ± 0.17
Gln19His	nitrile	1.8 ± 0.2		5.22 ± 0.16		8.00 ± 0.16
	aldehyde	0.011 ± 0.001		5.40 ± 0.18		8.11 ± 0.19
	substrate[b]			5.48 ± 0.06		8.09 ± 0.10

[a] Value not corrected for hydration of the aldehyde. [b] From Ménard et al. (1991a, 1995).

changes in pH optimum and width of pH−activity profiles for these mutants have been attributed to perturbations in the electrostatic environment of the active site and in the thiolate−imidazolium ion pair stability. This is particularly evident for the Gln19Glu mutant, where the pH profile with the peptide nitrile is not bell-shaped and displays an additional pK_a of 5.63 attributable to ionization of Glu19. In addition, the mutations caused decreases in affinity for the peptide nitrile. The K_i value of 0.84 μM for inhibition of wild-type enzyme increased to 11.9, 9.1, and 45 μM for Gln19Ala, Gln19Glu^0, and Gln19Glu^-, respectively, corresponding to 11−54-fold decreases in affinity. With Gln19His, the K_i value increased only by a 2-fold factor, to 1.8 μM.

Different results were obtained with the aldehyde inhibitor Ac-Phe-Gly-CHO. For the mutants Gln19Ala and Gln19His, the pK_a values describing the pH dependency of inhibition are similar to those observed for the peptide nitrile or for substrate hydrolysis (Table 1). However, the mutations do not cause an increase in K_i but instead result in a slight increase in affinity of the enzyme for the inhibitor, confirm-

Table 2: Pre-Steady-State Kinetic Parameters for Inhibition of Mutant and Wild-Type Papain by Ac-Phe-Gly-CHO

enzyme	k_1[a] (10^5 M^{-1} s^{-1})	k_2 (10^{-3} s^{-1})	K_i^{calc} [a,b] (nM)
papain	5.2 ± 1.9	19.4 ± 1.0	37
Gln19Ala	11.8 ± 2.4	25.7[c]	21.8[c]
Gln19Glu			
pH 5.0	3.5 ± 0.9	2.8 ± 2.4	8.0
pH 6.0	3.5 ± 0.9	6.0 ± 3.3	17
Gln19His	10.7 ± 2.1	7.9 ± 2.8	7.4

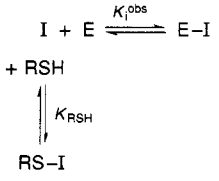
[a] Value not corrected for hydration of the aldehyde. [b] K_i^{calc} calculated from k_1 and k_2 values ($K_i^{calc} = k_2/k_1$). [c] k_2 could not be accurately determined and was calculated from k_1 and the K_i value obtained previously (Table 1).

ing our previous result (Ménard et al., 1991a). The most important difference, however, is with the Gln19Glu mutant (Figure 2c). The pH profile for inhibition of Gln19Glu by Ac-Phe-Gly-CHO is bell-shaped and is described by pK_a values different from those observed with the nitrile inhibitor or with a substrate (Table 1). In addition, the Gln19Glu mutant is the enzyme that binds the peptide aldehyde inhibitor the most tightly, with a K_i of 5.0 nM.

For Ac-Phe-Gly-CHO, the rate constants k_1 and k_2 for formation and breakdown of the enzyme−inhibitor complex could be determined and were obtained for wild-type and mutant papain. The results reported in Table 2, which are not corrected for hydration of the aldehyde [peptide aldehydes are 90% hydrated in solution (Lewis & Wolfenden, 1977a)], are in agreement with literature values (Frankfater & Kuppy, 1981). As can be seen with all enzymes, k_1 is relatively high, and the slow inhibition process is attributable to the low k_2 rate and to the low concentration of inhibitor in solution. Even though the precision of the data reported in Table 2 is not very high, the results indicate that mutation of Gln19 does not affect in a significant manner the k_1 or k_2 rates. Therefore, the lack of effect on K_i is not the result of compensating effects on k_1 and k_2. Values of K_i calculated from measured k_1 and k_2 rates (Table 2) are in agreement with those obtained from steady-state measurements (Table 1).

An important factor for comparing cysteine protease inhibition by peptide aldehydes and nitriles is the relative intrinsic affinities of these compounds for a thiol group. The principle of one of the approaches used in the present study to provide this information is outlined in Scheme 4. The

Scheme 4



dissociation constant for the enzyme−inhibitor complex was measured as described previously in presence of concentrations of a small thiol compound, varying between 0 and 300 mM. The dissociation constant K_{RSH} of the complex formed between the inhibitor and the thiol compound can be obtained from eq 5 by linear regression of the data.

$$K_i^{app} = K_i^{obs}(1 + [RSH]/K_{RSH}) \quad (5)$$

Table 3: Dissociation Constants for Reaction of Peptide Aldehyde and Nitrile Inhibitors with Small Thiol-Containing Nucleophiles

	K_{RSH}[a] (mM)	
inhibitor	2-ME	3-MPA
Ac-Phe-Gly-CHO[b]	1.3 ± 0.3	1.0 ± 0.2
MeOCO-Phe-Gly-CN	15 ± 2	260 ± 10[c]

[a] Dissociation constant for the complex between the inhibitor and the thiolate anion, obtained by correcting the value obtained at pH 9.0 for the pK_a values of the thiol group of 2-ME and 3-MPA. [b] Value not corrected for hydration of the aldehyde. [c] Determined from ^{13}C NMR experiments.

The experiments were carried out at pH 9.0, to ensure that an important fraction of the thiol compounds is present as the thiolate anion, which is a much better nucleophile and also a better model to compare to attack by the active site thiolate anion of Cys25 in papain. The neutral thiol was considered to be nonreactive compared to the thiolate form, and the results given in Table 3 for the two model thiol compounds 2-ME and 3-MPA have been corrected for the pK_a of the thiol group (experimentally determined pK_a = 9.7 for 2-ME and 10.1 for 3-MPA). For the aldehyde, the K_{RSH} values of 1.3 and 1.0 mM obtained with 2-ME and 3-MPA, respectively (not corrected for hydration of the aldehyde), are in the range of what has been reported for hemithioacetal dissociation (Frankfater & Kuppy, 1981; Burkey & Fahey, 1983; Moon & Hanzlik, 1987). The dissociation constants are higher for the thioimidates formed with MeOCO-Phe-Gly-CN. With 2-ME, a value of 15 mM was obtained for K_{RSH}. However, 2-ME can form chelates with nitriles (Lee et al., 1989), and therefore, K_{RSH} for 2-ME might be underestimated. With 3-MPA, where this possibility does not exist, very little effect on K_i^{app} was observed up to 300 mM of 3-MPA, indicating $K_{RSH} > 22$ mM, and ^{13}C NMR was used as an alternate method to measure K_{RSH} for the peptide nitrile. The ^{13}C resonances of the free nitrile (117 ppm) and of the thioimidate adduct with 3-MPA (181.6 ppm) were used to calculate K_{RSH}, and the result is given in Table 3. The same experiment was performed with 2-ME as the thiol compound, and a value of $K_{RSH} = 9 \pm 3$ mM was obtained, in agreement within experimental error with the kinetically determined value. It can be seen that the dissociation constant for the complex formed between 3-MPA and the peptide aldehyde Ac-Phe-Gly-CHO is approximately 260-fold lower than that for the complex with the nitrile compound. By comparison, the dissociation constant for the hemithioacetal formed between papain and Ac-Phe-Gly-CHO is lower than that for dissociation of the thioimidate formed with MeOCO-Phe-Gly-CN by a factor of 21.

DISCUSSION

The oxyanion hole of cysteine proteases contributes to catalysis by stabilizing inherently unstable transition states in the reaction pathway for substrate hydrolysis (Ménard et al., 1991a, 1995). For the mutation of Gln19 to Ala, Glu, and His, the transition state was estimated to be destabilized by 1.8−3.4 kcal/mol for hydrolysis of the substrate CBZ-Phe-Arg-MCA (Figure 3). On the other hand, the affinity of papain for a peptide aldehyde inhibitor has been shown to be almost unaffected by mutation of Gln19 of the oxyanion hole. As shown in Figure 3, the mutations introduced in

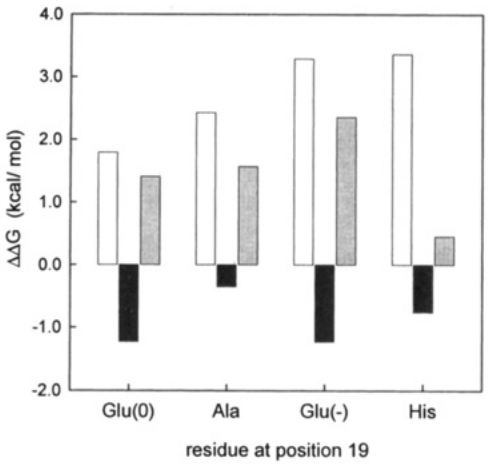


FIGURE 3: Comparison of the change in interaction energy upon mutation of residue Gln19 for a substrate (transition state, white bar), a peptide aldehyde (hemithioacetal, black bar), and a peptide nitrile (thioimidate, gray bar). For the inhibitors, $\Delta\Delta G = -RT \ln(K_i(\text{wild type})/K_i(\text{mutant}))$; for the substrate, $\Delta\Delta G = -RT \ln([k_{cat}/K_M(\text{mutant})]/[k_{cat}/K_M(\text{wild-type})])$.

the present study in fact resulted in a small but significant increase in stability of the hemithioacetal adduct (0.4−1.2 kcal/mol). In that respect, the hemithioacetal formed between papain and a peptide aldehyde cannot be considered a good model of the transition state for substrate hydrolysis.

For reactions with a substrate, significant negative charge character is considered to develop on the carbonyl oxygen of the scissile bond in the transition state. With the aldehyde inhibitor Ac-Phe-Gly-CHO, experimental data support a neutral hemithioacetal adduct with papain (Frankfater & Kuppy, 1981; Mackenzie et al., 1986), and this difference in charge state could explain the lack of effect observed upon mutation of Gln19 with the peptide aldehyde. However, an argument against this hypothesis is provided by the thioimidate adduct, which is stabilized through interaction with Gln19 despite the fact that it does not place a negatively charged atom but instead a neutral NH group in the oxyanion hole. One might consider the possibility of the hemithioacetal hydroxyl group being located outside of the oxyanion hole, as suggested by Mackenzie et al. (1986). However, the crystal structure of a complex formed between papain and the peptide aldehyde leupeptin clearly shows that the oxygen atom of the hemithioacetal is located in the oxyanion hole (Schröder et al., 1993). The dissociation constant K_i for inhibition of papain by leupeptin was determined and found to decrease slightly when residue Gln19 is replaced by alanine (data not shown), similar to the result with Ac-Phe-Gly-CHO. For the serine proteases, a class of enzymes that is believed to have a catalytic mechanism similar to that of cysteine proteases (Kraut, 1977), crystallography and NMR experiments have shown that the hemiacetal complex formed with a peptide aldehyde can exist in two configurations, where the oxygen atom of the hemiacetal is respectively in the oxyanion hole or located in hydrogen bonding distance of the active site histidine residue (Delbaere & Brayer, 1985; Ortiz et al., 1991). Both configurations of the hemithioacetal might also be possible with the cysteine protease papain; however, only one configuration was observed in the papain−leupeptin structure, possibly due to the conditions used to generate the crystals. Alternatively, the formation of a papain−aldehyde complex might involve a number of steps, where two tetrahedral adducts are generated with the hydroxyl group located either in or out of the oxyanion hole (Frankfater & Kuppy, 1981). The results of the present study suggest that in such a mechanism, the intermediate involved in the rate-limiting step would not be stabilized by oxyanion hole interaction with Gln19.

Inhibition of papain by a peptide nitrile occurs through the formation of a thioimidate adduct (Brisson et al., 1986; Moon et al., 1986; Liang & Abeles, 1987). This adduct is structurally closer to the acyl-enzyme than to the putative tetrahedral transition state in the mechanism for substrate hydrolysis (Figure 1). However, mutation of Gln19 was found to cause important increases in K_i, and thioimidate adducts with the papain mutants Gln19Ala and Gln19Glu are less stable by 1.4−2.4 kcal/mol. It can be seen from Figure 3 that the effect of oxyanion hole mutations on the stability of the thioimidate adduct follows a trend similar to that observed for hydrolysis of the substrate CBZ-Phe-Arg-MCA. One exception to this trend is seen with the mutant Gln19His. The decrease in affinity for the peptide nitrile with this mutant is the lowest of the four mutant forms, while the effect on substrate hydrolysis is the most important. It must be noted that conformational and ion pair stability perturbations have been suggested for the Gln19His mutant (Ménard et al., 1995), and these perturbations might affect substrate hydrolysis and thioimidate stability in different manners. The effect of Gln19 mutations on the pH−inhibition profiles with the peptide nitrile is also almost identical to the effect on pH−activity profiles for substrate hydrolysis. For the peptide aldehyde, a major difference is observed with the mutant Gln19Glu (Figure 2c). This difference cannot be attributed to hydration of the aldehyde, since hydration is independent of pH between 3.5 and 9.0 (Frankfater & Kuppy, 1981). Even though our data cannot provide any direct explanation for this difference, the presence of additional pK_a values suggests that the mechanism for formation of the hemithioacetal might be more complex and involve a number of steps. A pH-dependent change in the rate-determining step in the Gln19Glu mutant could lead to kinetic pK_a values different from those observed in the free enzyme for substrate hydrolysis. In particular, the pK_a of 5.04 in the acid limb was arbitrarily attributed to pK_2 in Table 1 because the ionization it describes leads to an increase in inhibitory activity. However, this pK_a is in fact closer to the pK_2' value reported for inhibition by the peptide nitrile and might not correspond to pK_2. The effects of the mutations on the K_i values and on the pH−inhibition profiles clearly show, however, that with respect to the influence of oxyanion hole mutations the inhibition of papain by peptide nitriles is a process closer to that of substrate hydrolysis than the inhibition by the corresponding aldehydes.

The potency of peptide aldehydes as inhibitors of cysteine proteases has often been interpreted as an indication that hemithioacetals are analogous to the putative tetrahedral transition state in the catalytic pathway for substrate hydrolysis. It must be noted, however, that the value of K_{RSH} with 3-MPA reported in Table 3 is 260-fold lower with Ac-Phe-Gly-CHO than that with MeOCO-Phe-Gly-CN. If a correction for hydration of the aldehyde is introduced (Lewis & Wolfenden, 1977a), we find that formation of the hemithioacetal is favored over formation of the thioimidate by a factor of 2050 in solution, and the intrinsic affinity of the carbon center of the inhibitor for the thiolate is a key

factor in determining the relative inhibitory activities of peptide aldehydes and nitriles. In fact, the ratio of K_i values for papain inhibition is only 21, in favor of the aldehyde (165 after correction for aldehyde hydration), and compared to the adducts in solution, the papain thioimidate is actually stabilized to a greater extent by the enzyme than the corresponding papain hemithioacetal. The extra stabilization observed with the thioimidate might be a result of oxyanion hole interaction, since mutation of Gln19 has been shown to destabilize the thioimidate adduct, while the effect is opposite and small with the hemithioacetal. Part of the stabilization provided by the enzyme is attributable to the interactions present in the initial Michaelis complex formed by the noncovalent association of the enzyme with the inhibitor. This initial complex is usually relatively weak [dissociation constant, ~1 mM (Frankfater & Kuppy, 1981)], and involves interactions with the peptide portion of the inhibitor. This leads, however, to a major decrease in entropy for the formation of the covalent adduct from the Michaelis complex and contributes to the stabilization effect of the enzyme. The fact that linear relationships between $\log(K_i)$ for inhibitors and $\log(k_{cat}/K_M)$ for substrates are observed with inhibitors of widely different hybridization and geometry at the reactive center suggests that the interactions of the peptide portions of the inhibitors and substrates might be the main contributor to the relationship. Therefore, a linear correlation between $\log(K_i)$ and $\log(k_{cat}/K_M)$ for inhibitors and their corresponding substrates does not necessarily confer "transition state properties" to enzyme–inhibitor adducts. Interestingly, in one of the original papers reporting inhibition of papain by peptide aldehydes, Westerik and Wolfenden (1972) had cautioned against the premature conclusion that papain–aldehyde complexes were transition state analogs.

The results of the present study brings additional arguments against the consideration of hemithioacetals as transition state models for cysteine protease-catalyzed reactions. It must be noted that the hemithioacetal adduct is not a "true" transition state but a stable intermediate. In the reaction pathway for substrate hydrolysis represented in Figure 1, the hemithioacetal might compare better to the tetrahedral intermediate (THI) than to the transition states. However, the transition state in the conversion of the aldehyde to this hemithioacetal adduct is also not destabilized by oxyanion hole mutations, as indicated by the effect of the mutations on the rates of formation and breakdown of the adduct (k_1 and k_2), and must also be different from that of an enzyme–substrate reaction. The energy diagram for the reaction pathway of Figure 1 is illustrated in Figure 4. For papain-catalyzed hydrolysis, bond breaking in THI is considered to be rate-determining (O'Leary et al., 1974), and thus formation of TS_2 represents the highest energy barrier. The effect of oxyanion hole mutations on the rate of hydrolysis of CBZ-Phe-Arg-MCA indicates that the energy level of TS_2 increases in the mutants, i.e., TS_2 is stabilized by oxyanion hole interactions. If the hemithioacetal (HTA) is considered analogous to THI, the results obtained with the peptide aldehyde would indicate that the THI is either not affected or slightly stabilized by replacement of Gln19. However, no experimental data on the interaction of the oxyanion hole with a true THI are available to verify this hypothesis. Based on structural similarities between the thioimidate (TI) and acyl-enzyme (EA), the influence of the Gln19 mutations on

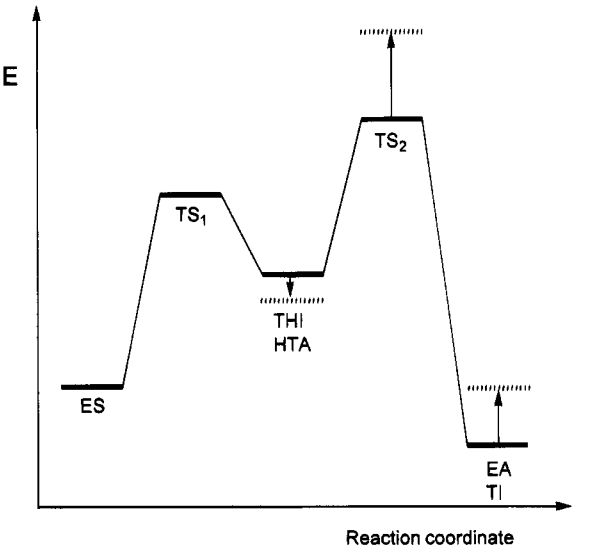


FIGURE 4: Energy diagram for substrate hydrolysis reactions catalyzed by cysteine proteases. ES, Michaelis complex; TS_1 and TS_2, transition states; THI, tetrahedral intermediate; HTA, hemithioacetal; EA, acyl enzyme; TI, thioimidate. The dashed lines represent the energy level of a Gln19 mutant enzyme, and the arrows indicate the direction and relative magnitude of the effect of the mutation from the wild-type levels.

thioimidate stability in papain could be considered to support a stabilization of the acyl-enzyme through oxyanion hole interactions. It has been proposed that acyl-enzymes are stabilized relative to simple thiol esters in solution by interaction with the enzyme (Hinkle & Kirsch, 1971). There is, however, a major difference between acyl-enzymes and thioimidates: the NH group of the thioimidate can act as a donor as well as an acceptor of a hydrogen bond, while the carbonyl oxygen of the acyl-enzyme can act only as an acceptor of two hydrogen bonds. Differences must therefore exist in the binding modes of acyl-enzymes and thioimidates in the active site of cysteine proteases.

The hypothesis that both TS_2 and possibly EA are stabilized by oxyanion hole interactions while THI is not (Figure 4) can be extended to infer that the nature of the highest energy transition state in substrate hydrolysis reactions by cysteine proteases is closer to that of the acyl-enzyme than to that of the tetrahedral intermediate. However, the data reported in the present study could also be interpreted as an indication that the catalytic mechanism might be other than a simple stepwise process involving intermediates. Recent experimental evidence and theoretical studies have questioned the nature of the transition state and of the catalytic process involved in substrate hydrolysis reactions (e.g., Arad et al., 1990, 1993; Hanzlik et al., 1991). In particular, it has been suggested that a concerted mechanism without formation of an anionic tetrahedral intermediate might be operative in cysteine proteases (Arad et al., 1990, 1993). Unfortunately, the influence of oxyanion hole interaction in the binding affinity of peptide aldehydes and nitriles to papain cannot provide a definite answer to this question.

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