Contribution to Activity of Histidine−Aromatic, Amide−Aromatic, and Aromatic−Aromatic Interactions in the Extended Catalytic Site of Cysteine Proteinases[†]

Dieter Brömme,* Pierre R. Bonneau,[‡] Enrico Purisima, Paule Lachance, Sohini Hajnik, David Y. Thomas, and Andrew C. Storer

Pharmaceutical Biotechnology Sector, Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montreal, Quebec H4P 2R2, Canada

Received September 26, 1995; Revised Manuscript Received January 11, 1996[⊗]

ABSTRACT: Within the papain family of cysteine proteinases few other residues in addition to the catalytic triad, Cys25-His159-Asn175 (papain numbering), are completely conserved [Berti & Storer (1995) *J. Mol. Biol. 246*, 273−283]. One such residue is tryptophan 177 which participates in a Trp-His-type interaction with the catalytic His159. In all enzymes of this class for which a three-dimensional structure has been reported, an additional highly conserved tryptophan, Trp181, also interacts with Trp177 via an aromatic−aromatic interaction in which the planes of the indole rings are essentially perpendicular. Also, both indole rings participate as pseudo-hydrogen bond acceptors in interactions with the two side chain amide protons of Asn175. Clearly, the proximity of Trp177 and Trp181 to the catalytic triad residues His159 and Asn175 and their network of interactions points to potential contributions of these aromatic residues to catalysis. In this paper, using cathepsin S, a naturally occurring variant that has a phenylalanine residue at position 181, we report the kinetic characterization of mutations of residues 175, 177, and 181. The results are interpreted in terms of the side chain contributions to catalytic activity and thiolate−imidazolium ion-pair stability. For example, the side chain of Asn175 has a major influence on the ion-pair stability presumably through its hydrogen bond to His159. The magnitude of this effect is modulated by Trp177, which shields the His159−Asn175 hydrogen bond from solvent. The His159−Trp177 interaction also contributes significantly to ion-pair stability; however, Trp181 and its interactions with Asn175 and Trp177 do not influence ion-pair stability to a significant degree. The observation that certain mutations at positions 177 and 181 result in a reduction of k_{cat}/K_m but do not appear to influence ion-pair stability probably reflects the contributions of these residues to substrate binding.

Although cysteine and serine proteinases are members of evolutionary distant proteinase classes, they share some mechanistic similarities. Both enzyme classes form covalent acyl-enzyme intermediates and have similar active site geometries (Brocklehurst et al., 1987; Polgar, 1987; Kraut, 1977; Polgar & Halasz, 1982). The catalytic triad in cysteine proteinases is constituted of a cysteine, a histidine, and an asparagine residue, whereas the triad in serine proteinases is formed by a serine, a histidine, and an aspartate residue. Besides these similarities, both enzyme classes reveal clear differences in their mechanisms. In the free state, cysteine proteinases possess a stabilized ion-pair formed between the active site cysteine and histidine residues and may therefore be regarded as "activated" enzymes (Polgar, 1974). On the other hand, serine proteinases are unchanged in their ground state, and the charge separation occurs during the formation of a tetrahedral intermediate in which the positively charged imidazole of the histidine is stabilized by the negatively charged aspartyl residue (Kraut, 1977). The aspartyl residue plays a critical role in the structural orientation of the histidine ring to the serine side chain (Kraut, 1977; Kossiakoff & Spencer, 1981; Sprang et al., 1987; Zimmerman et al., 1991). Replacement of the aspartic acid residue by an asparagine or alanine in the serine proteinases trypsin and subtilisin results in a dramatic decrease of their activities by 4 orders of magnitude (Craik et al., 1987; Carter & Wells, 1988). Conversely, mutation of the asparagine residue in the catalytic triad of the cysteine proteinase papain to an alanine results in only a 150-fold drop in enzymatic activity. It has been suggested (Vernet et al., 1995) that the strict conservation of Asn175 in cysteine proteinases might result from a combination of both functional and structural constraints.

Although the formation of an ion-pair in free cysteine proteinases as compared to serine proteinases is facilitated by the substantially higher acidity of thiol groups as compared to hydroxyls, the unusual pK_a values of the ion-pair participants cannot be explained on this basis alone; the local environment in the active site must also be considered. For example, the negative charge on the cysteine residue (Cys25), located at the N-terminus of the L-I helix, is suggested to be stabilized by an α-helix dipole (Hol et al., 1978). The side chain of Asn175 is hydrogen-bonded via

[†] P.R.B. was supported by an Industrial Post-Doctoral Fellowship from the Natural Sciences and Engineering Research Council of Canada (NSERC). This is NRC Publication Number 39911.

* Correspondence should be addressed to this author at Arris Pharmaceuticals Co., 385 Oysterpoint Blvd., Suite 3, South San Francisco, CA 94080. Telephone: (415) 737-8600. Fax: (415) 737-8590.

[‡] Bio-Mega Industrial Research Fellow.

[⊗] Abstract published in *Advance ACS Abstracts,* March 1, 1996.

0006-2960/96/0435-3970$12.00/0

its amide oxygen to the active site His159, and this may help to orient the histidine to Cys25 (Baker & Drenth, 1987), thus promoting the ion-pair interaction. Additionally, interdomain interactions can be expected to have an influence on ion-pair stability since the active site histidine and cysteine residues are separately located on the two domains found in cysteine proteinase structures; e.g., Ser176 forms an interdomain hydrogen bond to Gln19 and stabilizes the ion-pair (Ménard et al., 1991b).

To date, little attention has been given to the shielding of the active site residues His159 and Asn175 by the highly conserved residues Trp177 and Trp181 (Kamphius et al., 1984). In contrast to the solvent-exposed active site histidine residue in serine proteinases (His64 in subtilisin; His57 in trypsin), the analogous His159 and its hydrogen bond to Asn175 in cysteine proteinases are mainly buried by the conserved Trp177. It has previously been shown that Trp177 contributes up to 46% to the fluorescence intensity of papain (Lowe & Whitworth, 1974) and that the protonated active site histidine residue (His159) can quench this contribution. This indicates a possible charge transfer interaction between the Trp177 and the His159, which in turn may help to stabilize the essential charge separation in the ion-pair of active papain (Evans et al., 1981). Recent results on an aromatic−histidine interaction in the ribonuclease barnase support this hypothesis (Loewenthal et al., 1992) in that such an interaction stabilizes the protonated form of the histidine, thereby increasing its pK_a value.

Little is known about the role of Trp181 in cysteine proteinases. With the exception of cathepsin S and a low-temperature-induced enzyme found in tomato fruit, this residue is entirely conserved as a tryptophan in all sequenced papain-like proteinases (Berti & Storer, 1995), and in X-ray structures of papain (Kamphius et al., 1984), actinidin (Baker, 1980), and cathepsin B (Musil et al., 1991) it is in close proximity to and perpendicularly oriented to Trp177, indicating an aromatic−aromatic interaction (Burley & Petsko, 1995). Both indole rings also participate as pseudo-hydrogen bond acceptors in interactions with the two side chain amide protons of Asn175 (Levitt & Perutz, 1988). In cathepsin S, the tryptophan residue normally found at position 181 is replaced by a phenylalanine residue (Wiederanders et al., 1992). Human lysosomal cathepsin S shares with papain a 45% sequence identity (Wiederanders et al., 1992), a similar symmetrical pH dependency, and also stability at slightly alkaline pH values (Brömme et al., 1993). In the tomato cysteine proteinase, a cysteine residue is found at the position equivalent to Trp181 of papain (Schaffer & Fischer, 1988). In addition, the adjacent residue, 182, is an arginine whereas in all other enzymes in this class including cathepsin S this position is occupied by a conserved glycine that participates in a type II turn. Together the two sequence differences found in the tomato proteinase suggest that the structure of this enzyme differs significantly in this region from other papain-like cysteine proteinases.

In order to determine the role of the highly conserved Asn175 and the aromatic residues, at positions 177 and 181, and their network of interactions on the catalytic activity and on the ion-pair stability in cysteine proteinases, appropriate mutants of cathepsin S were generated, expressed in yeast, and evaluated by kinetic analysis.

EXPERIMENTAL PROCEDURES

Materials. Restriction endonucleases were obtained from New England Biolabs. T4 DNA ligase, T4 DNA polymerase, and thiopropyl-Sepharose 6B were purchased from Pharmacia, Sweden. Oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer. Z-Val-Val-Arg-MCA[1] and Z-Phe-Arg-MCA were synthesized as previously described (Brömme et al., 1989). Z-Phe-Arg-pNA was purchased from Bachem, Inc., Switzerland.

Mutagenesis and Expression. The gene encoding human cathepsin S was cloned into the modified yeast shuttle vector pVT100-U to create plasmid YpCS3 as described previously (Brömme et al., 1993). The *Escherichia coli* strain MC1061 was used as host for plasmid manipulations. Mutagenesis was performed by the method of Kunkel (1985), using the BioRad Muta-GeneTM kit. The oligonucleotides used for mutagenesis were (the underlined bases code for the mutated amino acids in positions 175, 177, and 181) as follows:

W177F: 5′CTT GTG AAG AAC AGC TTC GGC CAC AAC3′
W177L: 5′TGG CTT GTG AAA AAC AGC CTA GGC CAC3′
W177A: 5′TGG CTT GTG AAA AAC AGC GCC GGC CAC3′
W177Y: 5′GTG AAA AAC AGC TAC GGC CAC AAC TTT3′

F181W: 5′TGG GGC CAC AAC TGG GGT GAA GAA GG3′
F181L: 5′AAC AGT TGG GGC CAC AAC TTG GGT GAA GAA GG3′
F181A: 5′TGG GGC CAC AAC GCC GGC GAA GAA GG3′

W177A/F181A: 5′GTG AAA AAC AGC GCG GGC CAG AAC GCT GGT GAA GG3′

N175A: 5′TGG CTT GTG AAA GCA AGC TGG GGC3′
N175A/W177A: 5′TGG CTT GTG AAA GCT AGC GCC GGC CAC AAC TTT GGT3′
N175A/W177F: 5′TGG CTT GTG AAA GCT AGC TTT GGC CAC AAC TTT GGT3′

The mutations within the cathepsin S gene were verified by restriction enzyme analysis and DNA sequencing around positions coding for residues 175, 177, and 181.

The expression vectors for the mutants were transformed into the yeast strain BJ3501 (Ernst, 1986) (Matα pep4::HIS3, prb1-1.6R, his3 Δ200, ura3-52). The transformants were grown for 2 days in synthetic selective medium and 3 days in rich medium as described previously (Brömme et al., 1993).

Purification. The Trp177 and Phe181 mutants of human cathepsin S were purified as described for the wild-type enzyme (Brömme et al., 1993) with slight modification. The yeast cells of a 2 L culture grown in a Bacto-Lift system were lysed by three passages through a French press cell at 20 000 psi, combined with the concentrated medium supernatant, supplemented with 5 mM dithiothreitol, 5 mM EDTA, and 0.5 mM PMSF, and finally adjusted to pH 4.5 with 1 M acetic acid. The activation of the enzyme at 40 °C was monitored using a fluorogenic substrate assay (substrate: 10 μM Z-Val-Val-Arg-MCA) and stopped at maximal activity after 1−3 h. The activated lysate was clarified by centrifugation at 20000*g* and precipitated with ammonium sulfate at 80% saturation at room temperature. The activity containing pellet was redissolved in 100 mM sodium acetate/1 mM EDTA (pH 4.0) and clarified by centrifugation, and the supernatant was applied to a PDS (pyridyl disulfide)-activated thiopropyl-Sepharose 6B column (column volume: 2 mL). After consecutive washing of the column with 60 mL of 100 mM sodium acetate/1 mM EDTA, pH 4.0,

[1] Abbreviations: Z, benzyloxycarbonyl; MCA, 4-methyl-7-coumarylamide; pNA, *p*-nitroanilide; E-64, 1-(L-*trans*-epoxysuccinylleucylamino)-4-guanidinobutane; for the discussion of the interactions between proteinase and substrate, the nomenclature of Schechter and Berger (1967) was used.

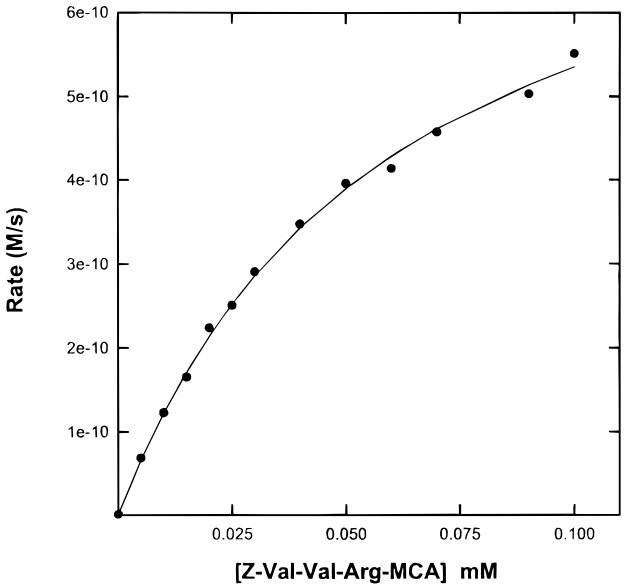
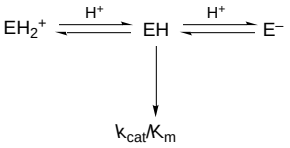


FIGURE 1: Michaelis−Menten plot of Z-Val-Val-Arg-MCA hydrolysis catalyzed by the cathepsin S mutant Trp177Tyr. The concentration of the mutant proteinase in the assay was 2.2 nM (other details are given in the text).

and 60 mL of 100 mM Tris-HCl/1 mM EDTA, pH 8.0, the activity was eluted with 20 mM cysteine/1 mM dithioerythritol in 100 mM Tris-HCl/1 mM EDTA, pH 6.5. This resulted in an approximately 1000-fold increase in specific activity for the mutants. For the low activity multiple mutant enzyme forms, the thiopropyl-Sepharose 6B column was consecutively washed with 10 bed volumes of 100 mM sodium acetate buffer/1 mM EDTA, pH 4.0, and 100 mM Tris-HCl/1 mM EDTA, pH 8.0, and the activity was immediately eluted with 20 mM cysteine/1 mM dithioerythritol in 100 mM Tris-HCl/1 mM EDTA, pH 6.5. All mutant preparations were monitored by immunoblotting following SDS−polyacrylamide gel electrophoresis as described (Brömme et al., 1993).

Kinetic Measurements. Initial rates of hydrolysis of methylcoumarylamide substrates (Z-Val-Val-Arg-MCA, Z-Phe-Arg-MCA) were monitored at 22 °C on a Cary 2200 spectrophotometer equipped with a fluorescence-detection unit at an excitation wavelength of 380 nm and a 440 nm cutoff filter, and representative data are shown in Figure 1. The hydrolysis of the *p*-nitroanilide substrate (Z-Phe-Arg-pNA) was followed at 405 nm using the same spectrophotometer. The values of k_{cat} and K_m were determined using nonlinear regression analysis (Leatherbarrow, 1987). The concentration of the wild-type enzyme and the mutant enzymes was determined by titration with E-64 (Barrett & Kirschke, 1981). Since all the mutants containing the Asn175Ala mutation were characterized by a decreased rate of inhibition with E-64, the mutants were allowed to incubate overnight with the epoxide inhibitor. To exclude misleading titration results obtained from an incomplete inhibition of the mutants with the low molecular weight E-64 inhibitor, the mutants were also titrated with the tight binding cysteine proteinase-specific protein inhibitor cystatin C under analogous conditions. Inhibitors of this type block activity by tightly binding to the substrate cleft without a direct contact to the catalytic residues (Machleidt et al., 1989). The binding constant toward the wild-type cathepsin S was estimated to be in the low picomolar range (Brömme et al., 1991). Both titrations gave comparable results; i.e., less than 3-fold differences in concentrations obtained by the two methods were observed, and given the low yield and the low activity of the mutants, these differences can be considered to be within experimental error.

pH profiles were obtained at substrate concentrations (Z-Val-Val-Arg-MCA) lower than the K_m value as described in Brömme et al. (1993). The following buffers were used: 90 mM sodium citrate (pH 3.0−5.8), 90 mM sodium phosphate (pH 5.8−8.0), and 90 mM sodium borate (pH 8.0−10.0). All buffers contained 1 mM EDTA and 0.4 M sodium chloride to minimize the variation in ionic strength. For the least-squares regression analysis of the pH−activity data using the Enzfitter program (Leatherbarrow, 1987), a three-protonation state model (Ménard et al., 1990) was used:



Precautions against Proteinase Contamination. To exclude the possibility of artifacts resulting from proteinase contamination during the preparation of the recombinant cathepsin S from the yeast host cell (strain BJ3501), two control preparations of yeast cell cultures were subjected to the same conditions and procedures as for the wild-type and mutant preparations: (1) preparation of BJ3501 transfected with the PVT100-U vector without the gene insert; and (2) preparation of BJ3501 transfected with YpCS4 (Cys25Ser mutant) (Brömme et al., 1993).

Prepration 1 gave no detectable activity against the sensitive fluorogenic substrates Z-Val-Val-Arg-MCA and Z-Phe-Arg-MCA. Preparation 2 gave an extremely low activity, at the detection limit of the fluorimeter used, against the substrate Z-Val-Val-Arg-MCA. The magnitude of the activity was 3 orders lower than the lowest activity reported in this paper. The trace activity was inhibitable by an excess of E-64 and by cystatin. Since the codon used for the serine mutant was generated by double nucleotide exchange in the codon of the wild-type, a partial back-mutation is unlikely, and it is possible that this trace of activity can be assigned to the Cys25Ser mutant itself. An endogenous cysteine endopeptidase in yeast is unknown.

Molecular Modeling. The starting point for the model building was an energy-minimized structure of the native enzyme of papain (Kamphius et al., 1984). The His159-Cys25 catalytic residues were assumed to be in the ion-pair state. For each of the mutations considered here, the mutant enzyme was energy-minimized, allowing all residues within 6.0 Å of the mutating residue(s) to move while keeping the rest of the protein fixed. This was further refined using 1 ps of molecular dynamics at 50 K in order to surmount low-energy barriers near the local minimum. The resulting structure from molecular dynamics was then energy-minimized to the bottom of its potential energy well. All calculations were carried out using AMBER forcefield (Weiner et al., 1986) as implemented in SYBYL 6.0 (Tripos Assoc., Inc.). The calculations were in vacuo simulations with a distance-dependent dielectric function to mimic solvent screening effects.

Table 1: Kinetic Constants for the Hydrolysis of Z-Val-Val-Arg-MCA by Wild-Type and Mutant Forms of Human Cathepsin S

enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
wild-type	15 ± 2	18 ± 2	830000 (4)[a]
Trp177Phe	0.7 ± 0.2	16 ± 1	44000 (3)
Trp177Tyr	1.5 ± 0.4	51 ± 9	29000 (2)
Trp177Leu	0.13 ± 0.01	77 ± 11	1690 (2)
Trp177Ala	0.9 ± 0.4	140 ± 10	6400 (2)
Phe181Trp	10.0 ± 0.3	19 ± 2	526000 (2)
Phe181Leu	0.020 ± 0.001	77 ± 8	260 (2)
Phe181Ala	0.3 ± 0.2	49 ± 8	6100 (3)
Trp177Ala/Phe181Ala	0.02 ± 0.01	47 ± 8	430 (3)
Asn175Ala	0.022 ± 0.007	77 ± 30	290 (2)
Asn175Ala/Trp177Ala	0.0020 ± 0.0002	130 ± 10	14 (2)
Asn175Ala/Trp177Phe	0.004 ± 0.001	170 ± 40	23 (2)

[a] The number in parentheses is the number of independent experiments.

Table 2: Kinetic Constants for the Hydrolysis of Z-Phe-Arg-MCA and Z-Phe-Arg-pNA by Wild-Type and Mutant Forms of Cathepsin S

enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
	Z-Phe-Arg-MCA		
wild-type	1.9 ± 0.2	22 ± 2	86000 (4)
Trp177Phe	0.17 ± 0.04	40 ± 7	4200 (3)
Trp177Tyr	0.34 ± 0.02	56 ± 2	6100 (2)
Trp177Leu	0.022 ± 0.002	67 ± 11	300 (2)
Trp177Ala	0.11 ± 0.03	112 ± 2	980 (2)
Phe181Trp	1.6 ± 0.1	32.5 ± 0.5	49000 (2)
Phe181Leu	0.0030 ± 0.0002	81 ± 8	40 (2)
Phe181Ala	0.07 ± 0.02	60 ± 10	1200 (3)
	Z-Phe-Arg-pNA		
wild-type	1.2 ± 0.04	100 ± 20	12000 (2)
Trp177Phe	0.27 ± 0.01	117 ± 12	2300 (2)
Trp177Ala			750 (2)

[a] The number in parentheses is the number of independent experiments.

RESULTS

Using site-directed mutagenesis, the tryptophan residue in position 177 has been replaced by a phenylalanine, tyrosine, leucine, or alanine residue, and the kinetic parameters for the hydrolysis of both a di- and a tripeptide substrate (Z-Phe-Arg-MCA, Z-Val-Val-Arg-MCA) have been determined (Tables 1 and 2). Conservative replacement of the Trp177 in cathepsin S by a phenylalanine or a tyrosine results in a 20−30-fold decrease in the second-order rate constant k_{cat}/K_m (Figure 2). This drop is mainly caused by 10−20-fold decrease in k_{cat}, whereas the K_m value is essentially unchanged for the phenylalanine mutant and only 3-fold increased for the tyrosine mutant. Although it cannot be generalized for cysteine proteinases that acylation is the rate-limiting step in the hydrolysis of amide substrates, it seems likely that the binding of the bulky MCA-leaving group is not severely affected by these mutations. In contrast, nonconservative mutations (Trp177, Leu, Ala) result in a significant increase in the K_m values by comparison with the wild-type enzyme (Table 1). These data indicate a dependency on the size and/or hydrophobicity of the residue at position 177 for the measured K_m values which may be interpreted as a decreased affinity of the enzyme for the substrate.

The mutation of Phe181 to a tryptophan residue has only a small effect on the K_m and on the k_{cat} values for both MCA substrates (Tables 1 and 2). Also, no difference between

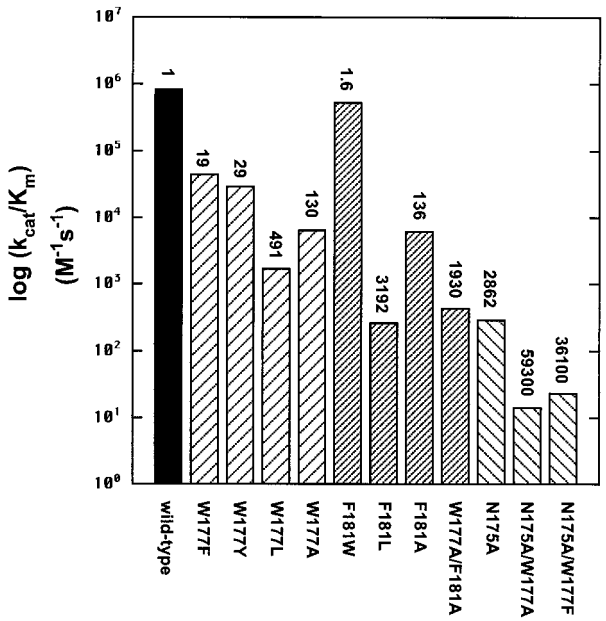


FIGURE 2: Comparison of k_{cat}/K_m values for the hydrolysis of Z-Val-Val-Arg-MCA by human cathepsin S and its mutants at positions 175, 177, and 181. The numbers on the top of the columns represent the ratio of $(k_{cat}/K_m)_{wt}/(k_{cat}/K_m)_{mut}$.

the inhibition of this mutant and the wild-type enzyme by cystatin C could be detected (D. Brömme, unpublished results). The replacement of Trp181 by a phenylalanine in cathepsin S has been confirmed in cathepsin S sequences of four different species (human, bovine, rat, and mouse; Wiederanders et al., 1991, 1992; Shi et al., 1992; Petanceska & Devi, 1992; unpublished sequence for the mouse enzyme from D. Steiner). The removal of the aromatic side chain has a relatively larger effect on k_{cat} values than on K_m values (Tables 1 and 2), with the largest changes being observed for the Trp181Leu mutant.

To evaluate whether mutations at positions 177 and 181 have a mutual influence or act independently from each other, double mutants have been produced (Carter et al., 1984; Horowitz, 1987; Horovitz et al., 1991). If changes at positions A and B in a protein are independent, then the sum of both effects should be the same as for the corresponding double mutant. If not, then mutual interactions between the residues occur, or structural pertubation with an effect on the activity takes place. Additivity is normally observed when the sites of multiple mutants are distant from each other (Wells, 1990). This method was recently successfully used for coordinated amino acid replacements in an interdomain interface in papain (Vernet et al., 1992). It is preferable for such experiments to compare the second-order rate constants since this constant is insensitive to possible changes in the rate-determining step or nonproductive binding modes introduced by the mutations. The results for the single mutants Trp177Ala and Phe181Ala and for the double mutant Trp177Ala/Phe181Ala show that the effects on the k_{cat}/K_m value are not additive (Table 3), suggesting that residues 177 and 181 interact.

The elimination of the hydrogen bond between Asn175 and His159 in cathepsin S in the Asn175Ala mutant results in a 2700-fold decrease in the second-order rate constant (k_{cat}/K_m) for the hydrolysis of the specific cathepsin S substrate Z-Val-Val-Arg-MCA. The drop in activity is mainly due to an approximately 700-fold decrease in k_{cat}. However, the

Table 3: Comparison of the Single Mutants Trp177Ala, Phe181Ala, and N175Ala with Their Double Mutants Trp177Ala/Phe181Ala, Asn175Ala/Trp177Ala, and Asn175Ala/Trp177Phe

	single mutant		product,		
	Trp177Ala	Phe181Ala	*sum*	double mutant	coupling energy
$(k_{cat}/K_m)_{wt}/(k_{cat}/K_m)_{mut}$	130	140	18200	1960	
$\Delta\Delta G$[a]	11.9	12.1	*24.0*	18.6	−5.4
	single mutant		product,		
	N175Ala	Trp177Ala	*sum*	double mutant	coupling energy
$(k_{cat}/K_m)_{wt}/(k_{cat}/K_m)_{mut}$	2862	130	372060	59286	
$\Delta\Delta G$	19.7	12.0	*31.7*	27.2	−4.5
	single mutant		product,		
	N175Ala	Trp177Phe	*sum*	double mutant	coupling energy
$(k_{cat}/K_m)_{wt}/(k_{cat}/K_m)_{mut}$	2862	19	54378	36087	
$\Delta\Delta G$	19.7	7.3	*27.0*	26.0	−1

[a] $\Delta\Delta G$ was calculated according to $\Delta\Delta G = -RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$.

Table 4: pK_a Values of pH−Activity Profiles for the Hydrolysis of Z-Val-Val-Arg-MCA by Wild-Type, Trp177, Phe181, and Asn175 Mutant Forms of Human Cathepsin S

enzyme	pK_a1	pK_a2	pK_a'1	width	midpoint
wild-type	4.48 ± 0.03	7.86 ± 0.03		3.38	6.17
Trp177Phe	4.51 ± 0.05	7.77 ± 0.04		3.26	6.14
Trp177Tyr	4.35 ± 0.01	7.75 ± 0.03	6.2 ± 0.3	3.39	6.06
Trp177Leu	4.80 ± 0.01	7.22 ± 0.03		2.42	6.01
Trp177Ala	5.14 ± 0.07	7.90 ± 0.05		2.72	6.52
Phe181Trp	6.65 ± 0.03	7.82 ± 0.04		3.17	6.23
Phe181Leu	4.76 ± 0.05	7.68 ± 0.05		2.92	6.22
Trp181Ala	4.52 ± 0.05	7.78 ± 0.05		3.26	6.15
Trp177Ala/Phe181Ala	4.52 ± 0.01	8.48 ± 0.09		3.96	6.50
Asn175Ala	4.72 ± 0.02	6.91 ± 0.07		2.19	5.82
Asn175Ala/Trp177Ala	4.49 ± 0.01	7.54 ± 0.09		3.05	6.02
Asn175Ala/Trp177Phe	5.1 ± 0.1	7.18 ± 0.01		2.08	6.14

elimination of the aromatic side chain of residue Trp177 (mutant Trp177Ala) and, therefore, a probable exposure of the hydrogen bond between Asn175 and His159 to the solvent decrease the k_{cat}/K_m only by 130-fold with a 17-fold decrease in k_{cat}. This indicates that Trp177 at most has only a partial influence on the stability of the hydrogen bond between Asn175 and His159 in cathepsin S. A series of mutations at position 177 (Trp177Phe, -Tyr, -Leu; Table 4) showed that tryptophan 177 increases the basicity of the active site histidine by increasing its pK_a, thereby enhancing the nucleophilicity of the catalytic cysteine. Additionally, it is likely that Trp177 decreases the rotational flexibility of the active site histidine. To distinguish between the hydrogen bridge shielding effect and the more direct effect on the histidine by Trp177, two double mutants Asn175Ala/Trp177X (X = Ala, Phe) were designed and analyzed. If the main effect of Trp177 on activity is through the shielding of the hydrogen bond between Asn175 and His159, then the single mutant Asn175Ala and the double mutant Asn175Ala/Trp177Ala should behave in a similar way since in both cases there is no hydrogen bond to protect. The k_{cat}/K_m of the double mutant drops 20-fold compared to the Asn175Ala mutant or 60 000-fold when compared to the parent enzyme, indicating that the role of Trp177 is not only in the physical shielding of the hydrogen bond. This conclusion is supported by the double mutant Asn175Ala/Trp177Phe which is characterized by a conservative mutation in position 177. Although the side chain in residue 177 is reduced in size only by the pyrrole part of the indole ring of tryptophan 177, the k_{cat}/K_m value decreases for this double mutant by the same order of magnitude (35 000-fold) as for the Asn175Ala/Trp177Ala mutant.

The lowest coupling energy was determined for the Asn175Ala/Trp177Phe mutant which reveals essentially additivity, e.g., an independent behavior of the two mutated residues (Table 3). The phenylalanine residue in position 177 has obviously no direct influence on the stability of the hydrogen bond between Asn175 and His159. This is not surprising since computer modeling studies on the papain structure reveal that this hydrogen bond is still sufficiently protected by a phenylalanine residue in position 177. The mutant Asn175Ala/Trp177Ala displays a coupling energy of −4.5 kJ/mol (Table 3), suggesting less additivity and a partly overlapping effect of the two sites of mutation on the activity of cathepsin S. The complete removal of the side chain of residue 177 exposes the hydrogen bond to the solvent which may partly destabilize this bond.

To obtain information on the influence of mutations at residues Trp177 and Phe181 on the ion-pair stability of cathepsin S, the pK_a values of the pH profiles for the mutants were determined (Table 4). The unusual pK_a values for the active site histidine and cysteine residues (Cys pK_a 4−4.5; His pK_a 8−8.5) have been shown to be a prerequisite for the formation of an thiolate−imidazolium ion-pair (Creighton & Schamp, 1980; Lewis et al., 1981) at neutral pH. Furthermore, the distance between the histidine and the cysteine residue has a large influence on the ion-pair stability (Pickersgill, 1988). Ménard et al. (1991a) could demonstrate that the values of pK_a1 and pK_a2, the pK_as that respectively

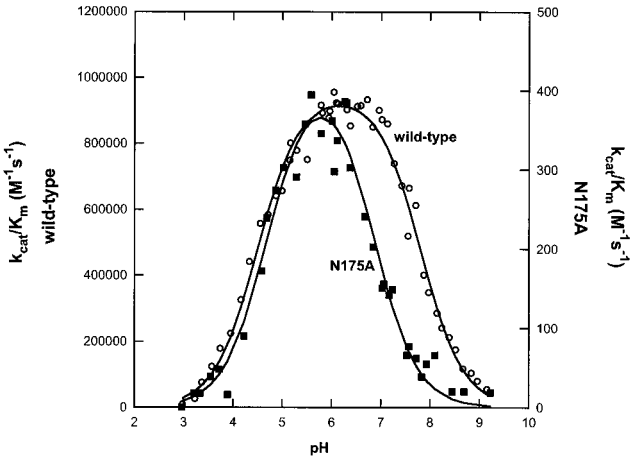


FIGURE 3: pH dependence of k_{cat}/K_m for the Asn175Ala mutant in comparison to the wild-type enzyme of human cathepsin S. k_{cat}/K_m values were determined at each pH by measuring the initial rate of Z-Val-Val-Arg-MCA hydrolysis and by dividing by enzyme and substrate concentrations.

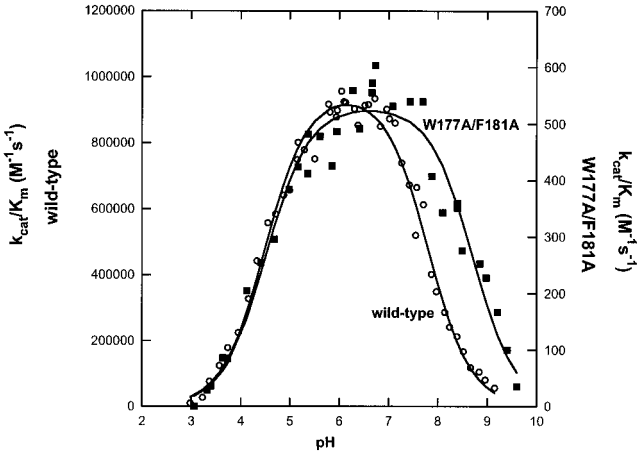


FIGURE 4: pH dependence of k_{cat}/K_m for the Trp177Ala/Trp181Ala double mutant in comparison to the wild-type enzyme of human cathepsin S.

define the acidic and basic limbs of the enzyme's pH−activity profile, can be affected by small conformational or environmental modifications and that variations of pK_a1 and pK_a2 reflect changes in ion-pair stability. According to the model, a destabilization of the ion-pair would result in a decrease in K_5^{int} (the equilibrium constant between the ion-pair and its neutral form) and in turn a narrowing of the pH profile.

The width of the pH profiles (pK_a2 − pK_a1) is not changed for the conservative mutations Trp177Phe and -Tyr, indicating that no detectable destabilization of the ion-pair occurs (Table 4). Interestingly, for the mutant Trp177Tyr, an additional pK_a of 6.17 is observed, and the activity of the higher pH form of the enzyme, i.e., above 6.17, is approximately double that of the lower pH form (data not shown). It is possible that this additional pK_a is due to the phenolate of the tyrosine which is shifted by the microenvironment in the active site to this unusual low value. In model peptides, the pK_a of the phenolate in tyrosine is only a little higher than that of cysteine (Fersht, 1985). The nonconservative mutations Trp177Leu and -Ala result in a drop in the width of the corresponding pH profiles by 0.9 and 0.6 pH units, respectively, which can be regarded as a considerable ion-pair destabilization. With the exception of the Phe181Leu mutant, none of the Phe181 mutants alter the width of the profile. This indicates that the stability of the ion-pair is more influenced by Trp177 than by Phe181. Compared to the wild-type enzyme, the widths of the pH profiles for the mutant Asn175Ala (Figure 3) and for the double mutant Asn175Ala/Trp177Phe are decreased by 1.14 and 1.25 pH units (Table 4), again indicating a significant destabilization of the ion-pair. However, the width of the pH profile is in the range of the parent enzyme for the Asn175Ala/Trp177Ala mutant.

Since the pH profile of the Trp177Ala mutant (Table 4) has a marked shift in its pK_a1 to a higher pH value and the Phe181Ala mutant does not exhibit an alteration of its pH profile, it was predicted that the pH profile of the double mutant Trp171Ala/Phe181Ala should resemble more that of the Trp177Ala mutant. Surprisingly, an even broader pH profile than that of the wild-type enzyme was determined (Figure 4), indicating some unknown compensating effects.

DISCUSSION

From the alignment of over 100 full or partial papain-like cysteine proteinase sequences (Berti & Storer, 1995), it can be seen that in addition to 4 half-cystines only 6 other residues are fully conserved. These residues (Gln19, Cys25, His159, Asn175, Trp177, and Gly185) form a cluster in the enzyme active sites and comprise the following: the catalytic triad residues, Cys25, His159, and Asn175; the oxyanion hole side chain, Gln19; and Trp177 and Gly185, whose functions, and hence the rationale for their conservation, have not been discussed at length in the literature. Gly185 is situated at the $i + 2$ position of a conserved type 1′ β turn, and the structural constraints placed on this position can readily account for the conservation of this residue. The conservation of Trp177 is probably closely linked to residue 181. This residue is highly conserved; i.e., a tryptophan residue is found at position 181 in all known papain class cysteine proteinase sequences with the exception of cathepsin S and a tomato enzyme which contain, respectively, either a phenylalanine or a cysteine residue at this position. In addition, in all enzymes of this class for which a three-dimensional structure has been reported and for which structural coordinates are available, the side chains of Trp177 and Trp181 are interacting through an aromatic−aromatic-type interaction (Figure 5). The planes of the indole rings are essentially perpendicular, and the centroids of the two indole six-membered rings are separated by 5.1 Å (Table 5). In addition, both indole rings participate as pseudo-hydrogen bond acceptors in interactions with the two side chain amide protons of Asn175. One proton is situated 2.4 Å above the plane of the Trp177 benzene on the perpendicular through the centroid of that ring. The second amide proton is situated 2.4 Å along a line through the centroid of the benzene ring of Trp181 that defines an angle of 73° with the plane of the ring. A further interaction between the side chains of Trp177 and His159 also exists. In this interaction, the centroids of the five- and six-membered rings of the indole are 4.8 Å and 5.0 Å from the centroid of the imidazole ring, respectively, and the planes of the two ring systems intersect at an angle of 34°. A similar Trp−His interaction that results in an elevated pK_a of the histidine side chain has been reported by Loewenthal et al. (1992) for the enzyme barnase. Clearly, the functional roles of residues 175, 177, and 181 are potentially both complex and highly coupled.

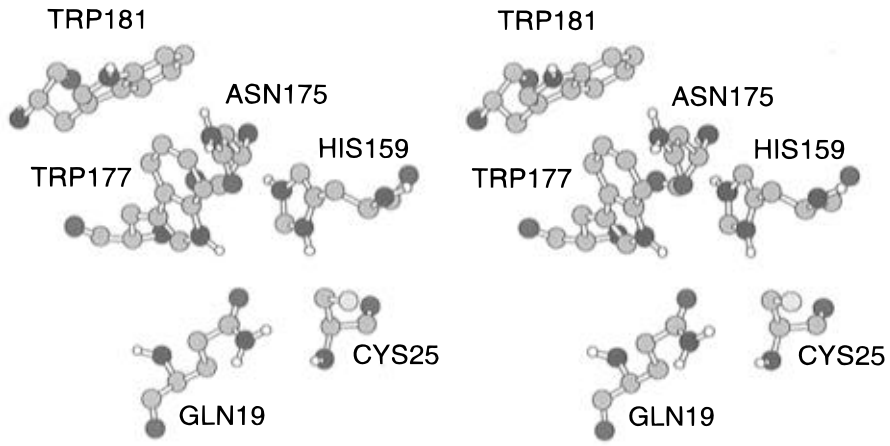


FIGURE 5: Stereo diagram showing the relative orientations of the side chains of residues Gln19, Cys25, Asn175, Trp177, and Trp181 in the structure of papain (Kamphius et al., 1984).

Table 5: Distances (Å) and Angles (Degrees) Derived from the Modeling Study of Cathepsin S Wild-Type and Mutant Forms

		distances (Å) for enzyme form		
from	to centroid of side chain benzene ring of residue	wild-type	Phe181Trp	Trp177Phe
closest side chain NH of Asn175	177	2.3	2.4	2.7
	181	2.6	2.4	2.3
centroid of side chain imidazole ring of His159	177	5.1	5.0	4.6
	181	8.3	8.1	
centroid of side chain benzene ring of residue 181	177	5.0	5.2	5.7

	angles (deg) for enzyme form		
angles between planes defined by side chain rings of residues	wild-type	Phe181Tcp	Trp177Phe
159 and 177	34	34	29
159 and 181	72	72	68
177 and 181	103	103	96

Thus, it can be anticipated that mutations at positions 175, 177, and 181 may affect the activity of cysteine proteinases through one or a combination of effects; however, a naturally occurring variant at position 181, cathepsin S and its mutants, provides valuable insight into the roles of these residues and their influences on the catalytic residues with which they interact.

Asparagine 175. The geometries of the catalytic triads in cysteine (Cys-His-Asn) and serine proteinases (Ser-His-Asp) are very similar (Garavito et al., 1977); however, nonconservative mutation of the Asp residue to Ala for the serine proteinase subtilisin and to Ser for trypsin resulted in approximately 10^4-fold reductions in enzymatic activity (Carter & Wells 1988; Corey et al., 1992). The apparently greater contribution of the Asp residue to the catalytic activity of serine proteinases over the Asn residue in cysteine proteinases probably reflects basic differences in the catalytic mechanisms of the two classes of enzyme (Polgar & Asboth, 1986). For serine proteinases, the formation of the transition state and tetrahedral intermediate is accompanied by charge separation, and it has been suggested that the negative charge on the aspartate contributes to this process and hence catalysis through electrostatic stabilization (Warshel et al., 1989). In cysteine proteinases, charge separation in the form of an ion-pair is already present in the ground state, and generation of the transition state and tetrahedral intermediate causes only a rearrangement of the charges. It has been suggested that the asparagine residue of the catalytic triad is of importance for the stabilization of the ion-pair form of the catalytic cysteine and histidine residues largely by a contribution to the maintenance of these active site residues in a conformation favorable for catalysis rather than a significant contribution through electrostatic stabilization (Drenth et al., 1975). This role for Asn175 was originally proposed based on the examination of the crystal structures of cysteine proteinases and the H-bond that is formed between the side chain amide oxygen of Asn175 and the NHϵ of His159. This H-bond is approximately collinear with the His159 Cβ−Cγ bond, thus allowing rotation of the imidazole ring of His159 around this bond without breaking the Asn175−His159 H-bond. Based on this observation, it has been suggested that the role of Asn175 is to optimally orientate the His159 to enable it to participate in the various steps in the enzyme's catalytic path. In the resting stage of the enzyme, the His159 side chain would be positioned such that the thiol group of Cys25 is in the plane of the imidazole ring, thus promoting proton transfer from the cysteine to the histidine side chain. Following substrate binding, the protonated imidazole ring would rotate by approximately 30° to be positioned for the optimal donation of its proton to the leaving group of the substrate (Drenth et al., 1975). In contrast to the relatively solvent-exposed but more orientationally fixed histidine residue in serine proteinases, the potentially flexible histidine in cysteine proteinases of the papain family is shielded from bulk solvent by a conserved tryptophan residue at position 177. Additionally, the hydrogen bond formed between Asn175 and His159 is shielded from solvent by Trp177 and Trp181 (Baker & Drenth, 1987).

An investigation of the role of the asparagine residue in the catalytic triad of cysteine proteinases (Cys-His-Asn) has been previously reported (Vernet et al., 1995). In that study, the Asn175 of papain was replaced by an alanine which

resulted in a 150-fold decrease in k_{cat}/K_m. The corresponding Asn175Ala mutation in cathepsin S has a significantly greater effect on that enzyme's catalytic activity, i.e., 2800-fold (Table 1, Figure 1). This larger effect can possibly be rationalized on the basis of the differing environments of the Asn175 residues and hence differences in the magnitude of the contribution of the residue to the catalytic efficiency of the two enzymes rather than reflecting differing catalytic roles. Although the crystal structure of cathepsin S has not been reported, it is possible to predict a significant difference in the environment of the residue by comparison with papain.

The contribution of Asn175 to the stability of the catalytic site thiol−imidazolium ion-pair is reflected by the sensitivity of the enzyme's pH−activity profile to mutations at this site (Figure 3; Vernet et al., 1995). It has previously been shown that factors influencing the catalytic ion-pair stability of cysteine proteases in addition to affecting the intrinsic activity of the enzymes will also result in a modification of the width of their pH−activity profiles (Ménard et al., 1991); i.e., destabilizing effects will result in narrower profiles while stabilizing effects will result in broader profiles. The model that relates ion-pair stability changes to measured changes in the activity and pH profile of a particular enzyme was developed for the characterization of mutations of noncatalytic residues (Ménard et al., 1991a). Complexities introduced as a result of mutating a catalytic residue such as Asn175 are discussed by Vernet et al. (1995). They conclude that although it is difficult to dissect out the relative effects of the Asn175Ala mutation in papain on both ion-pair stability and intrinsic activity, the model used is still consistent with changes in the width of the pH−activity profile reflecting changes in ion-pair stability. In this regard, it is interesting to note that the Asn175Ala mutation in papain results in a narrowing of the that enzyme's pH−activity profile by 1.58 pH units whereas the equivalent mutation in cathepsin S results in a 1.19 pH unit narrowing. Clearly, the same mutation in the two enzymes has a similar effect on the ion-pair stability.

Tryptophan 177. As stated above, both Trp177 and Trp181 potentially influence cysteine proteinase catalytic efficiency in a number of ways; for example, it has been suggested that they play a role in forming the S'_1 subsite of the binding site. It is known from studies with substrates (Ménard et al., 1993), inhibitors (Brömme & Kirschke, 1993), and nucleophiles (Schuster et al., 1992) that papain and other cysteine proteinases including cathepsin S show a preference for the binding of bulky aromatic groups in the S'_1 site, and it is suggested that these groups interact with the indole ring of Trp177. This suggestion is supported by the recently solved structure of the cysteine proteinase cathepsin B complexed with CA030, a specific epoxysuccinyl inhibitor (Turk et al., 1995). In this structure, the side chain of the inhibitor P'_1 isoleucine residue binds into a hydrophobic pocket defined by residues (cathepsin B numbering) Val176, Leu181, Met196, and Trp221. The latter residue is the equivalent of Trp177 in cathepsin S and papain. In addition, from the X-ray structure it can be seen that the tryptophan Nϵ1 atom and its associated proton form a hydrogen bond to the backbone carbonyl oxygen of the inhibitor P'_1 isoleucine. This involvement of Trp177 in forming part of the S'_1 subsite is further supported by the observation that on replacing the indole ring of Trp177 with smaller side chains, e.g., as in Trp177Phe and Trp177Ala (Tables 1 and 2), the activity of the enzyme drops in an essentially parallel fashion for the two MCA-based substrates but to a lesser degree for the smaller pNA substrate (Table 2). This indicates that the effect of mutations at position 177 on the activity of cathepsin S is influenced in part by the S'_1 enzyme−substrate interactions, indicating that the side chain of residue 177 contributes toward defining the S'_1 subsite specificity.

In Trp177, c2 is −12°, far from 90°; hence, from the modeling study, it is not possible to reposition the phenyl ring in Trp177Phe close to the space occupied by the benzene ring in Trp177 by a simple rotation about c1. Following a molecular dynamics minimization, the centroid of the phenyl ring in Trp177Phe is 2.7 Å from HN1 of Asn175, a significant increase from the 2.3 Å found in the wild-type enzyme, suggesting a weakening of the interaction between the aromatic residue at 177 and Asn175. It is interesting to note that the weakening of this interaction, as with the loss of a corresponding interaction between residue 181 and Asn175 (see below), does not result in a significant change in the width of the pH−activity profile and therefore there is no detectable decrease in the stability of the catalytic ion-pair.

The replacement of Trp177 by the nonaromatic amino acids alanine and leucine results in even larger reductions, approximately 100- and 400-fold, respectively, in the activity of cathepsin S (Tables 1 and 2) and also significant narrowing of the pH profile (Table 4). As with the Trp177Phe and Trp177Tyr mutants, the Trp171Ala and Trp177Leu mutations can be expected to result in significant effects on substrate binding due to the loss of hydrophobic and hydrogen-bonding interactions within the S'_1 subsite. Indeed, based on the changes in activity observed for the Trp177Phe and Trp177Tyr mutants, the decreases in activity of the Trp177Ala and Trp177Leu mutants can be expected to be in large part due to changes in the affinity of the enzyme for the substrate as a result of the mutations. The more pronounced decrease in activity as a result of the Trp177Leu mutation as compared with the Trp177Ala mutation is probably a result of the bulky nonplanar nature of the leucine side chain; i.e., the leucine side chain cannot be accommodated within the volume occupied by the aromatic side chain normally found at that position. Thus, the side chain of Trp177Leu can be expected to protrude significantly into the S'_1 binding site, thus introducing a steric interaction with substrate when bound. In addition, the nonplanar leucine side chain will structurally perturb the residues with which Trp177 normally interacts, i.e., His159 and Asn175, accounting for the narrower pH−activity profile obtained for this variant than is seen with the Trp177Ala mutant. Based on the results obtained with the Phe181Ala mutant described below, the pH−activity profile narrowing and the associated decrease in ion-pair stability observed with the Trp177Ala mutant cannot be explained in terms of the loss of the Asn175−Trp177 amide−aromatic interaction or the Trp177−Phe181 aromatic−aromatic interaction since the loss of the same or equivalent interactions with the Phe181Ala mutant had no detectable effect on the enzyme's pH−activity profile. These effects must therefore be due to loss of other Trp177 side chain properties, i.e., the His159−Trp177 histidine−aromatic

interaction or the shielding from bulk solvent afforded to the His159−Asn175 inter-side-chain hydrogen bond. The magnitude of the pH−activity profile change, −0.66 unit, is in line with the 0.6 pH unit change observed in the pK_a of a histidine residue (Loewenthal et al., 1992) in barnase when the tryptophan with which it interacts was mutated to an alanine. The introduction of a second mutant, Trp177Phe, into the Asn175Ala form of cathepsin S results in a further 13-fold drop in activity (Table 1), which is comparable to the 19-fold drop observed for the single Trp177Phe mutant, indicating that the effects of the two individual mutations are additive. However, the introduction of the second mutation Trp177Ala into the Asn175Ala mutant results in a 21-fold drop of activity, which is significantly less than the 130-fold drop obtained with the single mutant Trp177Ala. This latter result suggests that the effects on activity of the Trp177Ala and Asn175Ala are not additive and that a commonality exists in the influences of these two mutants on the activity of the enzyme. A possible explanation of this effect lies with the His159−Asn175 hydrogen bond in that the Asn175Ala mutation eliminates it and the Trp177Ala mutation probably weakens it through the resulting increased exposure of the hydrogen bond to bulk solvent.

As discussed above, in addition to a potential contribution to the S'_1 subsite through which it may interact with bound substrates and inhibitors, the side chain of Trp177 is involved in side chain−side chain interactions with His159, Asn175, and the Phe (cathepsin S) or Trp (other papain family cysteine proteases) at position 181. In contrast to Trp181, the mutation of Trp177 in cathepsin S to another aromatic residue, i.e., phenylalanine or tyrosine, results in a significant, approximately 25-fold, decrease in k_{cat}/K_m. This decrease is not accompanied by a detectable effect on the width of the pH profile of k_{cat}/K_m for the enzyme. From the modeling study, it can be seen (Table 5) that the Trp177Phe mutation retains the potential to participate in the equivalent histidine−aromatic, amide−aromatic, and aromatic−aromatic interactions with residues 159, 175, and 181, respectively, that the wild-type Trp177 is capable of forming. It is probable, however, that the strength of the interactions will differ somewhat. The modification of the S'_1 subsite as a consequence of the Trp177Phe and Trp177Tyr mutations will change both the shape and the hydrophobicity of the site in addition to eliminating the side chain Nϵ1 with its potential to hydrogen bond to the substrate's P'_1 carbonyl (the latter not being an issue with the substrates used in this study), probably resulting in less efficient binding of the substrate at this site. Hence, in light of no significant effects on the ion-pair stability, the impacts of the conservative Trp177Phe and Trp177Tyr mutations on the activity of the enzyme are most probably due to effects on substrate binding. As reported in Table 4, the introduction of a tyrosine residue at position 177 results in an additional pK_a of 6.17 in the pH dependency of k_{cat}/K_m for cathepsin S, with the higher pH form of the enzyme having approximately double the activity of the lower pH form. It is not possible, based on the available evidence, to identify the source of this additional pK_a; however, one candidate is a perturbed pK_a of Tyr177.

Tryptophan 181. From a computer modeling study using the structure of papain as a starting point, the direct replacement of the Trp181 indole ring by a Phe181 phenyl as in wild-type cathepsin S can be seen to result in the phenyl ring occupying the pyrrole region of the original tryptophan. In this orientation, the distance of the Asn175 HN2 to the centroid of the Phe181 phenyl is 2.4 Å, a distance comparable to that found in the crystal structures with Trp181. In addition, the modeled orientation of the Phe181 side chain allows for the maintenance of the 177−181 aromatic−aromatic side chain interaction. The equivalence of the Trp and Phe residues at position 181 can readily account for the high and comparable levels of enzymatic activity expressed by both wild-type and mutant Phe181Trp forms of cathepsin S (Tables 1 and 2). The importance of an aromatic residue is underscored by the 100-fold drop in k_{cat}/K_m upon mutating Phe181 to alanine (Tables 1 and 2). The even larger decrease in k_{cat}/K_m observed as a result of the Phe181Leu mutation (approximately 2500-fold) is probably due in part to the bulky nonplanar nature of the leucine side chain. From the modeling study, it can be seen that this side chain is unable to fit into the narrow slot that an indole or phenyl ring can readily occupy. With Phe181Leu, significant local structural perturbations can be expected that will influence Asn175 and Trp177 and in turn the residues with which they interact. This probably accounts for the narrower pH−activity profile observed for this mutant (Table 4), reflecting a decrease in ion-pair stability. It is also interesting to note that the mutations Phe181Trp and Phe181Ala do not result in a significant modification of the width of the pH profile of cathepsin S. This can be taken as an indication that the mutations do not influence to a significant degree the stability of the catalytic thiolate−imidazolium ion-pair. Consequently, since the elimination of the aromatic side chain in the mutant Phe181Ala results in a loss of the amide−aromatic interaction between residues 175 and 181 and also the aromatic−aromatic interaction between residues 177 and 181, neither of these two interactions can be considered as important for the stability of the ion-pair. The significant drop in activity observed for this mutation (approximately 100-fold, Tables 1 and 2) can possibly be explained on the basis of an effect on the substrate binding rather than a direct effect on the reactivity of the catalytic residues. A question, that for the time being must remain unanswered, is as follows: In light of the results described above, and in particular the relative insensitivity of the enzyme to the Phe181Trp mutation, why is a phenylalanine residue not found more frequently at position 181 of papain-like cysteine proteinase sequences?

The decreases in catalytic activity observed for the various mutations at positions 175, 177, and 181 have in part been explained in terms of decreases in the stability of the active site thiol−imidazolium ion-pair as evidenced by the narrowing of corresponding pH−activity profiles. However, as can be seen from Table 4 for the double mutant Trp177Ala/Phe181Ala, although the activity of the enzyme is decreased almost 2000-fold relative to wild-type, the pH−activity profile is broader than that of the wild-type enzyme by 0.58 pH unit. In addition, the Asn175Ala/Trp177Ala pH−activity profile is wider than expected based on the profiles of the two corresponding single mutants. These results suggest that for the lower activity mutants additional as yet unidentified factors must be taken into account when trying to relate catalytic activity to ion-pair stability through the use of perturbations to the width of the pH−activity profile of the enzyme.

REFERENCES

Baker, E. N. (1980) *J. Mol. Biol. 141*, 441−484.
Baker, E. N., & Drenth, J. (1987) in *Biological Macromolecules and Assemblies* (Jurnak, A., & McPherson, A., Eds.) Vol. 3, pp 313−368, John Wiley & Sons, Inc., New York.
Barrett, A. J., & Kirschke, H. (1981) *Methods Enzymol. 80*, 535−561.
Berti, P. J., & Storer, A. C. (1995) *J. Mol. Biol. 246*, 273−283.
Brocklehurst, K., Willenbrock, F., & Salih, E. (1987) in *Hydrolytic Enzymes* (Neuberger, A., & Brocklehurst, K., Eds.) Chapter 2, Elsevier, Amsterdam.
Brömme, D., & Kirschke, H. (1993) *FEBS Lett. 322*, 211−214.
Brömme, D., Steinert, A., Friebe, S., Fittkau, S., Wiederanders, B., & Kirschke, H. (1989) *Biochem. J. 264*, 475−481.
Brömme, D., Rinne, R., & Kirschke, H. (1991) *Biomed. Biochim. Acta 50*, 631−635.
Brömme, D., Bonneau, R. P., Lachance, P., Wiederanders, B., Kirschke, H., Peters, C., Thomas, D. Y., Storer, A. C., & Vernet, T. (1993) *J. Biol. Chem. 268*, 4832−4838.
Burley, S. K., & Petsko, G. A. (1995) *Science 229*, 23−28.
Carter, P., & Wells, J. A. (1988) *Nature 332*, 564−568.
Carter, P. J., Winter, G., Wilkinson, A. J., & Fersht, A. J. (1984) *Cell 38*, 835−840.
Corey, D. R., McGrath, M. E., Vasquez, J. R., Fletterick, R. J., & Craik, C. S. (1992) *J. Am. Chem. Soc. 114*, 4905−4907.
Craik, C. S., Roczniak, S., Largman, C., & Rutter, W. J. (1987) *Science 237*, 909−913.
Creighton, D. J., & Schamp, D. J. (1980) *FEBS Lett. 110*, 313−318.
Drenth, J., Swen, H. M., Hoogenstraaten, W., & Sluyterman, L. A. A. (1975) *Proc. K. Ned. Akad. Wet., Ser. C: Biol. Med. Sci. 78*, 104−110.
Ernst, J. F. (1986) *DNA 5*, 483−491.
Evans, B. L. B., Knopp, J. A., & Horton, H. R. (1981) *Arch. Biochem. Biophys. 206*, 362−371.
Fersht, A. (1985) in *Enzyme Structure and Mechanism*, p 156, W. H. Freeman and Company, New York.
Garavito, R. M., Rossman, M. G., Argos, P., & Eventoff, W. (1977) *Biochemistry 16*, 5065−5071.
Hol, W. G. J., van Dujinen, P. T., & Berendsen, H. J. C. (1978) *Nature 273*, 443−446.
Horovitz, A. (1987) *J. Mol. Biol. 196*, 733−735.
Horovitz, A., Serrano, L., & Fersht, A. J. (1991) *J. Mol. Biol. 219*, 5−9.
Kamphuis, I. G., Kalk, K. H., Swarte, M. B. A., & Drenth, J. (1984) *J. Mol. Biol. 179*, 233−256.
Kossiakoff, A. A., & Spencer, S. A. (1981) *Biochemistry 20*, 6462−6474.
Kraut, J. (1977) *Annu. Rev. Biochem. 46*, 331−358.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
Leatherbarrow, R. J. (1987) *Enzfitter*, Elsevier Biosoft, Cambridge, MA.
Levitt, M., & Perutz, M. F. (1988) *J. Mol. Biol. 201*, 751−754.
Lewis, S. D., Johnson, F. A., & Shafer, J. A. (1981) *Biochemistry 20*, 48−51.
Loewenthal, R., Sancho, J., & Fersht, A. R. (1992) *J. Mol. Biol. 224*, 759−770.
Lowe, G., & Whitworth, A. S. (1974) *Biochem. J. 141*, 503−515.
Machleidt, W., Thiele, U., Laber, B., Assfalg-Machleidt, I., Esterl, A., Wiegand, G., Kos, J., Turk, V., & Bode, W. (1989) *FEBS Lett. 243*, 234−238.
Ménard, R., Khouri, H. E., Plouffe, C., Dupras, R., Ripoll, D., Vernet, T., Tessier, D. C., Laliberte, F., Thomas, D. Y., & Storer, A. C. (1990) *Biochemistry 29*, 6706−6713.
Ménard, R., Khouri, H. E., Laflamme, P., Dupras, R., Vernet, T., Tessier, D. C., Thomas, D. Y., & Storer, A. C. (1991a) *Biochemistry 30*, 5531−5538.
Ménard, R., Plouffe, C., Khouri, H. E., Dupras, R., Tessier, D. C., Vernet, T., Thomas, D. Y., & Storer, A. C. (1991b) *Protein Eng. 4*, 307−311.
Ménard, R., Carmona, E., Plouffe, C., Brömme, D., Konishi, Y., Lefebvre, J., & Storer, A. C. (1993) *FEBS Lett. 328*, 107−110.
Musil, D., Zucic, D., Turk, D., Engh, R. A., Mayr, I., Huber, R., Popovic, T., Turk, V., Towatari, T., Katunuma, N., & Bode, W. (1991) *EMBO J. 10*, 2321−2330.
Petanceska, S., & Devi, L. (1992) *J. Biol. Chem. 267*, 26038−26043.
Pickersgill, R. W. (1988) *Protein Eng. 2*, 247−248.
Polgar, L. (1974) *FEBS Lett. 74*, 15−18.
Polgar, L. (1987) in *Hydrolytic Enzymes* (Neuberger, A., & Brocklehurst, K., Eds.) Chapter 3, Elsevier, Amsterdam.
Polgar, L., & Halasz, P. (1982) *Biochem. J. 207*, 1−10.
Polgar, L., & Asboth, B. (1986) *J. Theor. Biol. 121*, 323−326.
Schaffer, M. A., & Fischer, R. L. (1988) *Plant Physiol. 87*, 431−436.
Schechter & Berger (1967) *Biochem. Biophys. Res. Commun. 27*, 157−162.
Schuster, M., Kasche, V., & Jakubke, H. D. (1992) *Biochim. Biophys. Acta 1121*, 207−212.
Shi, G. P., Munger, J. S., Meara, J. P., Rich, D. H., & Chapman, H. A. (1992) *J. Biol. Chem. 267*, 7258−7262.
Sprang, S., Standing, T., Fletterick, R. J., Stroud, R. M., Finer-Moore, J., Xuong, N.-H., Hamlin, R., Rutter, W. J., & Craik, C. S. (1987) *Science 237*, 905−909.
Turk, D., Podobnik, Popovic, T., Katunuma, N., Bode, W., Huber, R., & Turk, V. (1995) *Biochemistry 34*, 4791−4797.
Vernet, T., Tessier, D. C., Khouri, H. E., & Altschuh, D. (1992) *J. Mol. Biol. 224*, 501−509.
Vernet, T., Tessier, D. C., Chatellier, J., Plouffe, C., Lee, T. S., Thomas, D. Y., Storer, A. C., & Ménard, R. (1995) *J. Biol. Chem. 28*, 16645−16652.
Warshel, A., Åqvist, J., & Creighton, S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5820−5824.
Weiner, S. J., Kollman, P. A., Nguyen, D. T., & Case, D. A. (1986) *J. Comp. Chem. 7*, 230−252.
Wells, J. A. (1990) *Biochemistry 29*, 8509−8517.
Wiederanders, B., Brömme, D., Kirschke, H., Kalkkinen, N., Rinne, A., Paquette, T., & Toothman, P. (1991) *FEBS Lett. 286*, 189−192.
Wiederanders, B., Brömme, D., Kirschke, H., v.Figura, K., Schmidt, B., & Peters, C. (1992) *J. Biol. Chem. 267*, 13708−13713.
Zimmerman, S. C., Korthals, J. S., & Cramer, K. D. (1991) *Tetrahedron 47*, 2649−2660.

BI9523015