Articles

Engineering of Papain: Selective Alteration of Substrate Specificity by Site-Directed Mutagenesis[†]

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ABSTRACT: The S_2 subsite specificity of the plant protease papain has been altered to resemble that of mammalian cathepsin B by site-directed mutagenesis. On the basis of amino acid sequence alignments for papain and cathepsin B, a double mutant (Val133Ala/Ser205Glu) was produced where Val133 and Ser205 are replaced by Ala and Glu, respectively, as well as a triple mutant (Val133Ala/Val157Gly/Ser205Glu), where Val157 is also replaced by Gly. Three synthetic substrates were used for the kinetic characterization of the mutants, as well as wild-type papain and cathepsin B: CBZ-Phe-Arg-MCA, CBZ-Arg-Arg-MCA, and CBZ-Cit-Arg-MCA. The ratio of k_{cat}/K_M obtained by using CBZ-Phe-Arg-MCA as substrate over that obtained with CBZ-Arg-Arg-MCA is 8.0 for the Val133Ala/Ser205Glu variant, while the equivalent values for wild-type papain and cathepsin B are 904 and 3.6, respectively. This change in specificity has been achieved by replacing only two amino acids out of a total of 212 in papain and with little loss in overall enzyme activity. However, further replacement of Val157 by Gly as in Val133Ala/Val157Gly/Ser205Glu causes an important decrease in activity, although the enzyme still displays a cathepsin B like substrate specificity. In addition, the pH dependence of activity for the Val133Ala/Ser205Glu variant compares well with that of cathepsin B. In particular, the activity toward CBZ-Arg-Arg-MCA is modulated by a group with a pK_a of 5.51, a behavior that is also encountered in the case of cathepsin B but is absent with papain. Results of this study suggest that sequence alignment of cysteine proteases coupled with the structural information that is available for papain can be used to achieve a better understanding of the molecular mechanism and specificity of structurally and functionally related cysteine proteases.

Papain is a cysteine protease isolated from the latex of tropical papaya fruit (*Carica papaya*). This enzyme has been extensively characterized (Baker & Drenth, 1987; Brocklehurst et al., 1987) and its three-dimensional structure has been determined at high resolution (Kamphuis et al., 1984). Cathepsin B, on the other hand, is a cysteine protease found in the tissues of many animals, including human liver (Barrett, 1977), for which no crystal structure is presently available. However, the complete or partial amino acid sequences of cathepsin B from many sources are available, and comparison of the aligned sequences for papain and cathepsin B suggests that both proteases have a similar folding pattern and three-dimensional structure (Kamphuis et al., 1985; Dufour, 1988). Furthermore, the mechanistic data gathered so far for cathepsin B suggests that it shares a common basic enzymatic mechanism with papain. Features of this mechanism include the formation of a thiolate–imidazolium ion pair involving the side chains of the catalytic-site cysteine and histidine residues (Polgâr, 1974; Lewis et al., 1976, 1981) and the presence of an acyl-enzyme intermediate in the catalytic pathway (Bajkowski & Frankfater, 1983).

From comparisons of the available cysteine protease structures and sequences, it can be seen that groups required for interacting with the backbone of the substrates are provided by the backbone atoms of the enzyme and are therefore conserved. However, differences exist between the regions of the enzyme that bind the substrates' side chains. If papain and cathepsin B do possess very similar three-dimensional structures, then from these differences it should be possible to predict the features that lead to their different substrate specificites. Schechter and Berger (1967) have shown that the active site of papain can be considered to consist of seven subsites (S_1–S_4 and S_1'–S_3'), each able to accommodate one amino acid residue of a substrate (P_1–P_4 and P_1'–P_3', respectively). The nature of the residue at position P_2 has been shown to be the most significant in terms of determining specificity, and it is well established that papain exhibits a strong catalytic preference for amino acids with a bulky nonpolar side chain (e.g., Phe, Tyr) at this position (Berger & Schechter, 1970). Examination of the crystal structures of chloromethyl ketone derivatives of papain published by Drenth et al. (1976) allows identification of the residues in the S_2 subsite whose side chains make the most intimate contacts with the P_2 side chain of the substrate. These residues are Pro68,[1] Val133, Val157, Asp158, and Ala160; they form a hydrophobic cavity in papain (Table I.) The corresponding residues in cathepsin B, obtained from the sequence alignment, remain hydrophobic and the nonpolar character of the S_2 subsite is therefore conserved. Accordingly, it has been shown that substrates with an aromatic group (i.e., Phe) at P_2 are

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[1] The amino acid numbering of papain is used throughout the text.

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Table I: Amino Acid Residues Whose Side Chains Are Involved in Substrate Binding in the S_2 Subsite of Papain and Corresponding Residues in Cathepsin B[a,b]

enzyme	amino acid number[c] 68	133	157	160	205[d]
papain	P	V	V	A	S
human cathepsin B	P	A	G	A	E
mouse cathepsin B	P	A	G	A	E
rat cathepsin B	P	A	G	A	E
bovine cathepsin B	P	A	G	A	E
tomato cysteine protease	M	A	V	A	E

[a] Sequences are from Cohen et al. (1986) for papain, Chan et al. (1986) for human, mouse, and rat cathepsin B, Meloun et al. (1988) for bovine cathepsin B, and Schaffer and Fisher (1988) for tomato cysteine protease. [b] The backbone carbon atoms of residue 158 also form part of the S_2 subsite. [c] The amino acid numbering of papain starting at Ile1 is used. [d] In papain, residue 205 is not directly involved in substrate binding.

good substrates of cathepsin B (Barrett, 1980). The specificity of cathepsin B is, however, somewhat different from that of papain in that substrates bearing a basic arginine group in the P_2 position also react well with cathepsin B while they are very poor substrates for papain (Barrett & Kirschke, 1981). Comparison of the amino acid sequences of papain and cathepsin B indicates that Ser205, located at the back of the hydrophobic cavity of papain, is replaced by a glutamic acid residue in cathepsin B (Table I). By a detailed study of the pH dependence of cathepsin B catalyzed hydrolyses of synthetic substrates, Khouri et al. (1991) have shown that the specificity of cathepsin B for basic residues in P_2 could be accounted for by the existence of an interaction between the guanidinium group of an arginine side chain and the carboxylate group of the Glu205 (Glu245 in cathepsin B numbering) side chain in the S_2 subsite of cathepsin B. However, no direct evidence for the existence of such an interaction has been found.

Protein engineering offers a unique method to investigate the specificity of an enzyme. By site-directed mutation of amino acids believed to be in contact with a substrate, the residues responsible for determining the specificity of an enzyme can be determined and their function evaluated. In the present case, on the basis of strong similarities between the two related enzymes papain and cathepsin B, we predicted that we should be able to introduce cathepsin B like specificity into papain by replacing residues in the S_2 subsite that are in contact with a substrate by the equivalent residues present in cathepsin B. Alteration of substrate specificity through protein engineering has been accomplished for a variety of enzymes [e.g., subtilisin (Estell et al., 1986; Wells et al., 1987a,b), tyrosyl-tRNA synthetase (Fersht et al., 1985), trypsin (Craik et al., 1985; Graf et al., 1987), carboxypeptidase Y (Winther et al., 1985), α-lytic protease (Bone et al., 1989), lactate dehydrogenase (Clarke et al., 1989), and alcohol dehydrogenase (Murali & Creaser, 1986)]. Few reports have dealt with changing the specificity of an enzyme to resemble that of another known enzyme (Wells et al., 1987a), particularly two enzymes from such different sources as the plant protease papain and the mammalian enzyme cathepsin B.

From a comparison of amino acid sequences for papain and cathepsin B and with the 3D structure of papain as a guide, the major differences in the S_2 subsites of the two enzymes can be localized at positions 133, 157, and 205 (see Table I and Figure 1). The mutations Val133 → Ala, Val157 → Gly, and Ser205 → Glu were therefore introduced in papain to produce the papain variant Val133Ala/Val157Gly/Ser205Glu.[2] Furthermore, by analogy with the tomato cysteine protease, where position 157 is occupied by a valine (Schaffer & Fischer, 1988), a second papain mutant was produced where Val133 and Ser205 were replaced with Ala and Glu, respectively, leaving Val157 unchanged. The resulting double mutant is designated as Val133Ala/Ser205Glu. This paper reports the kinetic characterization of these two variants of papain. More significantly, this study explores the possibility that the sequence alignment of plant and mammalian enzymes coupled with structural information on the plant enzyme papain could be used to achieve a better un-

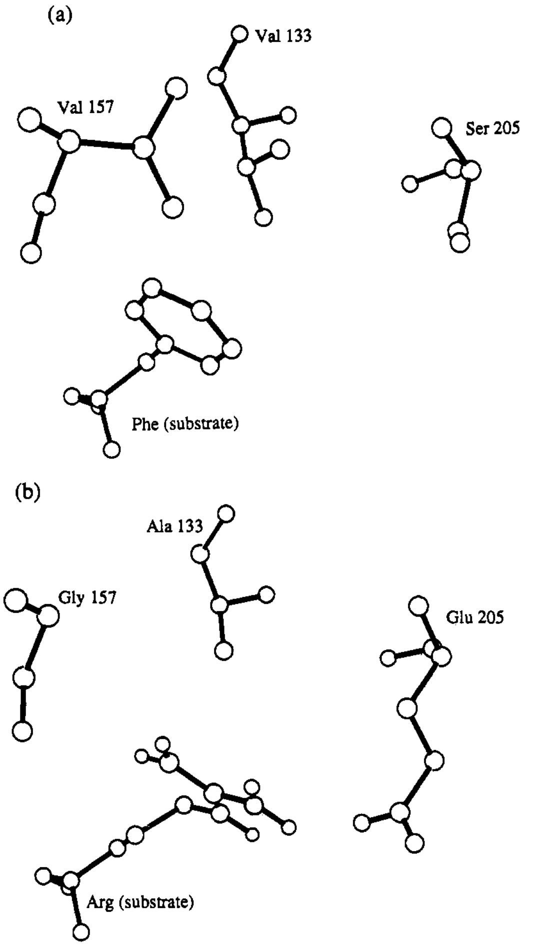

FIGURE 1: Computer modeling prediction of interactions in the S_2 subsite of the triple mutant Val133Ala/Val157Gly/Ser205Glu and, by analogy, cathepsin B. (a) Interactions in the S_2 subsite of papain between the Phe side chain of the CBZ-Phe-Ala-CH_2Cl inhibitor complexed to papain and residues Val133 and Val157 of the enzyme [from the crystal structure of Drenth et al. (1976)]. Residue 205 is included for comparison. (b) Model of the S_2 subsite of Val133Ala/Val157Gly/Ser205Glu. Residues 133, 157, and 205 of the structure shown in (a) have been replaced by Ala, Gly, and Glu, respectively, and the Phe side chain of the inhibitor has been replaced by that of an Arg residue to show the potential for interaction with the Glu205 side chain. The carbonyl, C_α, and C_β carbon atoms of the two structures have been superimposed. The torsional angles of the Glu and Arg side chains in (b) are within the range of values usually found for these side chains in proteins.

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[2] Abbreviations: Val133Ala/Val157Gly/Ser205Glu, triple mutant of papain; Val133Ala/Ser205Glu, double mutant of papain; CBZ-Phe-Arg-MCA, carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumarinyl-7-amide hydrochloride; CBZ-Arg-Arg-MCA, carbobenzoxy-L-arginyl-L-arginine 4-methylcoumarinyl-7-amide hydrochloride; CBZ-Cit-Arg-MCA, carbobenzoxy-L-citrullyl-L-arginine 4-methylcoumarinyl-7-amide hydrochloride; E-64, 1-[[(L-*trans*-epoxysuccinyl)-L-leucyl]amino]-4-guanidino)butane; MCA, 7-amino-4-methylcoumarin; oligo(s), oligodeoxynucleotide(s).

derstanding of the molecular mechanism and specificity of structurally and functionally related cysteine proteases. If this is the case, papain could serve as a template for the design of inhibitors aimed at related cysteine proteases of pharmacological importance such as cathepsins B, H, and L and the cysteine proteases of protozoan parasites (North et al., 1990; Shaw, 1990).

MATERIALS AND METHODS

Papain (2× crystallized suspension in sodium acetate) was obtained from Sigma Chemical Co. and was further purified as previously described (Sluyterman & Wijdenes, 1970). The mercurated form of papain thus obtained was activated by incubation with β-mercaptoethanol followed by gel filtration on Sephadex G-15. Cathepsin B, isolated and purified from human liver, was a generous gift from Dr. Sadiq Hasnain (Institute for Biological Sciences, National Research Council of Canada). The enzyme was activated by incubation for 1 h in 20 mM DTT at pH 6.0. The substrates CBZ-Phe-Arg-MCA and CBZ-Arg-Arg-MCA as well as the inhibitor E-64 were purchased from IAF Biochem International Inc., Laval, Québec. Mercurial agarose, thiopropyl-Sepharose 6B, and lentil lectin Sepharose 4B were from Pharmacia, Uppsala, Sweden.

Synthesis of CBZ-Cit-Arg-MCA. Carbobenzoxy-L-citrulline was prepared by the method of Bodansky and Birkhimer (1962). CBZ-Cit-Arg-MCA was synthesized by the methodology of Zimmerman et al. (1977). The dihydrochloride salt of L-arginine 4-methylcoumarinyl-7-amide (0.25 g, 6.21×10^{-4} mol) was dissolved in water and 1 equiv of *N*-methylmorpholine (0.068 mL) was added. The monohydrochloride salt, which precipitated out of solution, was filtered and dried. Carbobenzoxy-L-citrulline (0.192 g, 6.21×10^{-4} mol) was dissolved in dimethoxyethane and cooled to 0 °C. *N*-Methylmorpholine (0.068 mL) was added, followed by isobutyl chloroformate (0.90 mL, 6.21×10^{-4} mol). The solution was stirred for 30 min and the monohydrochloride salt of L-arginine 4-methylcoumarinyl-7-amide was added. This is only slightly soluble in dimethoxyethane, but after stirring overnight at room temperature no solid remains. The dimethoxyethane was removed in vacuo, and the residue was dissolved in water and washed with ethyl acetate. The water was removed and the product crystallized from methanol/ethyl acetate to give the monohydrochloride salt of CBZ-Cit-Arg-MCA (0.201 g, 49% yield). The product was characterized by ^{1}H NMR (in CD_3OD): δ 1.5–2.0 (m, 8 H, CH_2), 2.45 (s, 3 H, CH_3), 3.1–3.4 (m, 4 H, CH_2N), 4.1–4.5 (m, α-CH), 4.5–4.55 (m, α-CH), 5.1 (s, 2 H, CH_2Ar), 6.2 (s, 1 H, vinylic H), 7.2–7.35 (m, 5 H, ArH), 7.5 (d, 1 H, ArH), 7.7 (d, 1 H, ArH), 7.8 (s, 1 H, ArH). Molecular mass was confirmed by FAB mass spectra analysis [expected ($M^+ + 1$), 623.2941; observed, 623.2927].

Site-Directed Mutagenesis. Construction of the transfer vector IpDC127 containing a synthetic gene encoding the papain precursor (prepropapain) has been described elsewhere (Vernet et al., 1990). Site-directed mutagenesis was performed with mutant oligonucleotides priming synthesis on ssDNA templates enriched with uridine (Kunkel, 1985) in order to facilitate the selection of the mutant strand. The triple mutant was constructed in two independent steps. First, mutations Val157Gly and Ser205Glu were built simultaneously in a single in vitro reaction by mixing equimolar amounts of the mutagenic oligos 5′-GTGGTAACAAGGGTGACCATGCTG-3′ (Val157Gly) and 5′-GTTTGTACACCGAAAGTTTCTACCC-3′ (Ser205Glu) with IpDC127 ssDNA. Positive clones were identified by restriction enzyme analysis and showed the absence of both *Sal*I and *Xho*I sites diagnostic of the mutations. The ssDNA of double mutant Val157Gly,Ser205Glu was then used as template and was annealed to the Val133Ala mutagenic oligo 5′-CAGTTTCTGTAGCTCTAGAAGCGG-3′. The mutated papain gene in the resulting plasmid, IpDC156, was resequenced and no unexpected modification was found. The papain double mutant (Val133Ala/Ser205Glu) was constructed in a single step by using the two mutagenic oligos described above (Val133Ala and Ser205Glu). Construction of the recombinant baculovirus by cell-mediated homologous recombination was performed according to standard procedures (Summers & Smith, 1987). The identity of the recombinant baculovirus was verified by restriction enzyme mapping around the position coding for each of the three mutations after amplification of this region by the polymerase chain reaction (Tessier et al., 1991). *Spodoptera frugiperda* Sf9 insect cells were grown in spinner flasks and infected as described earlier (Vernet et al., 1990), except that the osmolarity of the Grace's medium used to wash the cells was maintained at 380 mosM by the addition of 2 mL of sterile 80% glycerol/L of medium. The secreted papain precursor mutant was activatable in vitro to the mature protein after incubation at 50 °C, pH 4.0, and was purified as described elsewhere (Ménard et al., 1991).

Kinetic Measurements. All kinetic experiments were performed at 25 °C as previously described (Ménard et al., 1990). The enzyme assay mixture at pH 6.0 consisted of 50 mM phosphate buffer, 0.2 M NaCl, 5 mM EDTA, and 10% CH_3CN, pH 6.0. Reactions with cathepsin B also contained 0.2 mM DTT in the assay mixture.

The kinetic parameters k_{cat}, K_M, and k_{cat}/K_M[3] for substrate hydrolysis at pH 6.0 were obtained by initial rate measurements and linear regression of the data to plots of s/v vs s. For pH–activity profiles, initial rates were determined at substrate concentrations such that $s \ll K_M$. k_{cat}/K_M values were obtained by dividing the initial rates measured at several pH values by the enzyme and substrate concentrations. The reaction mixture for pH–activity measurements is the same as for studies at pH 6.0, except the following buffers were used: 50 mM citrate for pH 3.0–5.7, 50 mM phosphate for pH 5.7–7.9, and 50 mM borate for pH 7.9–10.0.

The pH–activity profiles of papain are best described by model 1, in which three ionizable groups modulate the activity and only one form (HEH) of the enzyme is active (Ménard et al., 1990). For this model, the pH–activity profile is described by eq 1.

model 1

$$\begin{tikzcd} \mathrm{HEH_3} \arrow[r, leftrightharpoons, "K_1"] & \mathrm{HEH_2} \arrow[r, leftrightharpoons, "K_2"] & \mathrm{HEH} \arrow[r, leftrightharpoons, "K_3"] \arrow[d, "(k_{cat}/K_M)_1^{lim}"] & \mathrm{HE} \\ & & {} & \end{tikzcd}$$

$$k_{cat}/K_M = \frac{(k_{cat}/K_M)_1^{\,lim}}{\dfrac{[H^+]^2}{K_1K_2} + \dfrac{[H^+]}{K_2} + 1 + \dfrac{K_3}{[H^+]}} \qquad (1)$$

For the mutants of papain, the replacement of Ser205 by a glutamic acid has introduced an additional ionizable group that will be shown to modulate the activity of both Val133Ala/Val157Gly/Ser205Glu and Val133Ala/Ser205Glu. Ionization of this group is accounted for in model

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[3] k_{cat}/K_M represents an experimental value of the specificity constant while $(k_{cat}/K_M)_1^{lim}$ and $(k_{cat}/K_M)_2^{lim}$ are limiting values of the specificity constant for two different protonic forms of the enzyme obtained by nonlinear regression of pH–(k_{cat}/K_M) data to a specified equation.

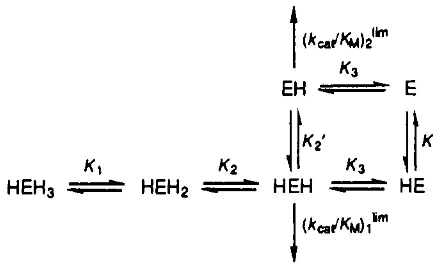
Table II: Kinetic Parameters for Hydrolysis of Substrates of the Type CBZ-P_2-Arg-MCA[a]

enzyme	P_2 residue	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($M^{-1}\ s^{-1} \times 10^{-3}$)	ratio[b]
Val133Ala/Ser205Glu	Phe	16.9 ± 7.6	0.191 ± 0.063	89 ± 23	8.0
	Arg	9.3 ± 3.6	0.94 ± 0.55	11.2 ± 3.3	1.0
	Cit	5.67 ± 0.75	1.296 ± 0.047	4.37 ± 0.49	0.4
Val133Ala/Val157Gly/Ser205Glu	Phe	2.78 ± 0.24	0.43 ± 0.22	7.4 ± 3.4	61
	Arg	0.79 ± 0.21	5.6 ± 1.6	0.120 ± 0.029	1.0
	Cit	0.42 ± 0.12	2.03 ± 0.32	0.205 ± 0.050	1.7
papain	Phe	41.6 ± 6.8	0.089 ± 0.006	464 ± 44	904
	Arg	1.18 ± 0.44	2.27 ± 0.75	0.514 ± 0.021	1.0
	Cit	8.14 ± 0.21	1.48 ± 0.10	5.50 ± 0.25	11
cathepsin B	Phe	32.8 ± 8.2	0.71 ± 0.23	47.1 ± 5.2	3.6
	Arg	29 ± 17	2.21 ± 0.43	13.1 ± 7.6	1.0
	Cit	48.3 ± 4.2	1.68 ± 0.27	29.1 ± 3.1	2.2

[a] Measurements were done at pH 6.0 except for wild-type papain, where the pH was 6.5. [b] Ratio is k_{cat}/K_M value relative to that with CBZ-Arg-Arg-MCA as substrate.

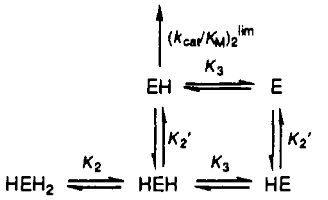
2, in which pK_2' reflects the ionization of Glu205 and two forms of the enzyme (HEH and EH) are active toward the substrates used in this study.

model 2



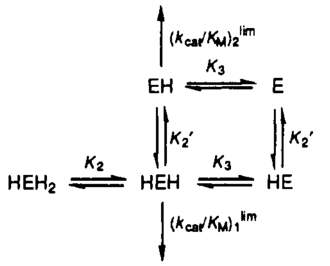
It was found, however, that, depending on the substrate, the pH dependence of activity can be described by simpler forms of model 2. These models (2a and 2b) are given with their corresponding equations, and they will be discussed in the Results section.

model 2a



$$k_{cat}/K_M = \frac{(k_{cat}/K_M)_2^{lim}}{\frac{[H^+]}{K_2'} + 1 + \frac{K_3}{[H^+]} + \frac{K_3}{K_2'}} \tag{2a}$$

model 2b



$$k_{cat}/K_M = \frac{(k_{cat}/K_M)_1^{lim}}{\frac{[H^+]}{K_2} + \left(1 + \frac{K_3}{[H^+]}\right)\left(1 + \frac{K_2'}{[H^+]}\right)} + \frac{(k_{cat}/K_M)_2^{lim}}{\frac{[H^+]^2}{K_2K_2'} + \left(1 + \frac{K_3}{[H^+]}\right)\left(1 + \frac{[H^+]}{K_2'}\right)} \tag{2b}$$

RESULTS

The wild-type and mutant enzymes have been characterized by using three synthetic substrates with different side chains in the P_2 position: CBZ-Phe-Arg-MCA, CBZ-Arg-Arg-MCA, and CBZ-Cit-Arg-MCA. The citrulline residue is isosteric to arginine but lacks the positive charge on the side chain, allowing evaluation of electrostatic contributions to the rates of hydrolysis. The kinetic parameters for hydrolysis of these substrates by wild-type papain, cathepsin B, and the Val133Ala/Ser205Glu and Val133Ala/Val157Gly/Ser205Glu mutants of papain are given in Table II. Papain exhibits a strong preference (904-fold) for a Phe over an Arg residue in the P_2 position of the substrate while this preference is much less with cathepsin B (3.6-fold). The mutants Val133Ala/Val157Gly/Ser205Glu and Val133Ala/Ser205Glu of papain, which were designed to reproduce the cathepsin B specificity in the S_2 subsite, show ratios of k_{cat}/K_M for CBZ-Phe-Arg-MCA over CBZ-Arg-Arg-MCA of 61 and 8.0, respectively, indicating, particularly in the case of the double mutant, that cathepsin B like specificity can be successfully introduced into papain. It must be noted, however, that with Val133Ala/Val157Gly/Ser205Glu, an important decrease in activity is observed for all three substrates used in this study compared with wild-type papain, while the Val133Ala/Ser205Glu mutant displays cathepsin B like specificity with a much smaller effect on the k_{cat}/K_M values for hydrolysis of CBZ-Phe-Arg-MCA or CBZ-Cit-Arg-MCA. Residue Val157 of papain therefore seems to be important for maintaining the overall activity of the enzyme.

From Table II it can be seen that the variation in specificity on proceeding from wild-type to Val133Ala/Ser205Glu papain is due to a combination of moderate effects on k_{cat} and K_M with the CBZ-Phe-Arg-MCA and CBZ-Arg-Arg-MCA substrates. The kinetic parameters for hydrolysis of CBZ-Cit-Arg-MCA are not significantly affected by the Val133 → Ala and Ser205 → Glu mutations. With the triple mutant Val133Ala/Val157Gly/Ser205Glu, however, the important decrease in activity can be attributed in a large part to lower values of k_{cat} in the mutant enzyme for all three substrates, including CBZ-Cit-Arg-MCA.

The relative S_2 subsite specificities of papain and cathepsin B are also reflected by differences in their pH–activity profiles (Khouri et al., 1991). For wild-type papain, the pH dependence of activity can be explained by model 1, where the enzyme can exist in four different ionization states, one of them (HEH) being active with the substrates used in this study (Ménard et al., 1990). The pH–activity profiles for wild-type papain have been reanalyzed under the conditions used in the present work (i.e., 10% CH_3CN), and the results are given in Table III. It must be noted that the values of pK_1, pK_2, and

Table III: Analysis of pH–Activity Profiles for Hydrolysis of CBZ-P_2-Arg-MCA by DM133/205 and Wild-Type Papain

enzyme	P_2 residue	model[a]	pK_1	pK_2	pK_2'	pK_3	$(k_{cat}/K_M)_1^{lim}$ (M^{-1} s^{-1} × 10^{-3})	$(k_{cat}/K_M)_2^{lim}$ (M^{-1} s^{-1} × 10^{-3})
Val133Ala/Ser205Glu	Phe	1	3.84 ± 0.66	4.44 ± 0.20		8.55 ± 0.13	92 ± 23	(92 ± 23)[b]
	Arg	2a		4.35 ± 0.38	5.51 ± 0.09	8.48 ± 0.09		14.9 ± 4.4
	Cit	2b		4.47 ± 0.37	5.59 ± 0.27	8.33 ± 0.25	1.86 ± 0.21	5.42 ± 0.61
papain	Phe	1	3.58 ± 0.29	4.54 ± 0.29		8.45 ± 0.02	482 ± 46	
	Arg	1	3.57 ± 0.40	4.43 ± 0.16		8.38 ± 0.07	0.530 ± 0.022	
	Cit	1	3.19 ± 0.24	4.58 ± 0.02		8.39 ± 0.01	5.73 ± 0.26	

[a] Minimum model describing the pH–activity profile. The parameters were obtained by nonlinear regression of the data to the corresponding equations as described in the text. [b] Models 1 and 2 cannot be differentiated if $(k_{cat}/K_M)_1^{lim} = (k_{cat}/K_M)_2^{lim}$. Since Glu205 does ionize with a pK_a of 5.5–5.6, as shown with the substrates where P_2 = Arg or Cit, the fact that the minimum model able to account for the influence of pH on hydrolysis of CBZ-Phe-Arg-MCA by Val133Ala/Ser205Glu is model 1 indicates that $(k_{cat}/K_M)_1^{lim} = (k_{cat}/K_M)_2^{lim}$ for this substrate. The value of $(k_{cat}/K_M)_2^{lim}$ in the table is therefore not obtained from the nonlinear regression procedure but inferred from these considerations.

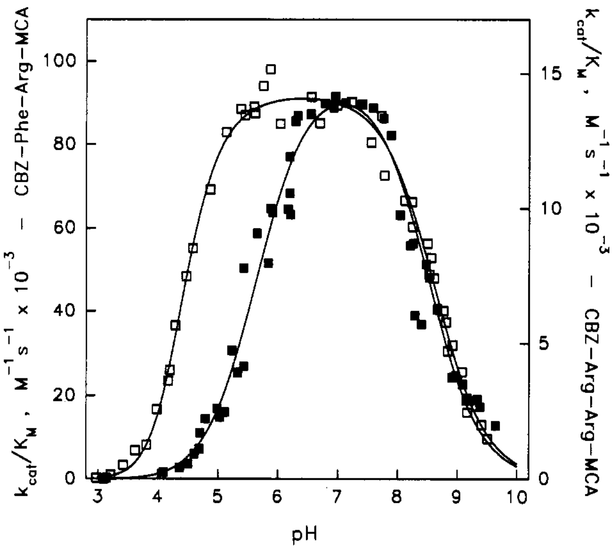


FIGURE 2: pH dependence of k_{cat}/K_M for hydrolysis of CBZ-Phe-Arg-MCA (□) and CBZ-Arg-Arg-MCA (■) by the Val133Ala/Ser205Glu variant of papain. The data were obtained as described in the text. The solid lines represent the best fit of the data to model 1 for CBZ-Phe-Arg-MCA and to model 2a for CBZ-Arg-Arg-MCA.

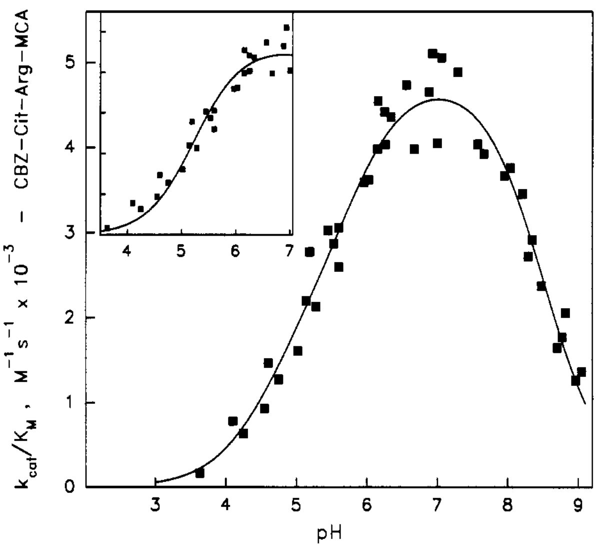


FIGURE 3: pH dependence of k_{cat}/K_M for hydrolysis of CBZ-Cit-Arg-MCA by the Val133Ala/Ser205Glu variant of papain. The data were obtained as described in the text. The solid line represents the best fit to model 2b. The insert corresponds to the best fit of the data to a model considering only one active form of the enzyme (only the low-pH region is shown for clarity).

pK_3 (obtained by nonlinear regression of the data to eq 1) are identical within experimental error for all three substrates. The corresponding profiles for the triple mutant Val133Ala/Val157Gly/Ser205Glu cannot be interpreted in the same way due to the very low activity of the mutant, particularly at low and high pH values. However, since the Val133Ala/Ser205Glu mutant is very active, it is possible to carry out a quantitative analysis of its pH–activity profiles.

The pH dependence of activity for hydrolysis of CBZ-Phe-Arg-MCA and CBZ-Arg-Arg-MCA by the Val133Ala/Ser205Glu mutant is illustrated in Figure 2. Hydrolysis of CBZ-Phe-Arg-MCA by this mutant displays a pH–activity profile very similar to those for wild-type papain, i.e., the profile can be described by model 1 and the pK_a's obtained by nonlinear regression are within experimental error identical with those found for papain (Table III). The value of $(k_{cat}/K_M)_1^{lim}$ is of course higher with wild-type papain than with the double mutant Val133Ala/Ser205Glu. The pH–activity profile for Val133Ala/Ser205Glu with the CBZ-Arg-Arg-MCA substrate is, however, very different from that observed for wild-type papain and resembles more closely the profile obtained for cathepsin B (Khouri et al., 1991). Since one of the mutations introduces an ionizable group (Glu) in the S_2 subsite of papain, model 1 has to be modified to take into account the possibility that this additional ionizable group could modulate the activity of the mutant enzyme toward certain substrates. Introduction of this additional ionizable group leads to model 2, where K_2' reflects ionization of the engineered Glu205 and where the enzyme can now exist in two active forms with Glu205 neutral (HEH) or ionized (EH). It was found during analysis of our data that the pH–activity profile with CBZ-Arg-Arg-MCA and the double mutant can be described by a simpler form of model 2, model 2a. In this model, the HEH form of the mutant enzyme is either inactive or has a very low activity that is undetectable under our assay conditions. As a consequence, the group with an ionization constant of K_1 does not appear to modulate the activity because the enzyme is already very weakly active at pH values where this group ionizes. Curve-fitting of the data for the CBZ-Arg-Arg-MCA substrate to eq 2a allows determination of the pK_a's, which are listed in Table III. It can be seen that pK_2 and pK_3 are very similar to those found in wild-type papain, and the pK_2' value of 5.51 is what one would expect for ionization of a glutamic acid residue in a hydrophobic region such as the S_2 subsite of the papain mutant.

For the hydrolysis of CBZ-Cit-Arg-MCA by Val133Ala/Ser205Glu (Figure 3), the profile seems to be "intermediate" between those for CBZ-Phe-Arg-MCA and CBZ-Arg-Arg-MCA, and the data cannot be described by either model 1 or model 2a since the enzyme form HEH also displays a significant activity toward the citrulline derivative. This is shown in the inset of Figure 3, where the best-fit curve obtained by considering only one active form of the enzyme is compared to the data. It can be seen that the data systematically fall over the calculated curve at low pH and under the curve at high pH, a clear indication that more than one active form of the enzyme has to be considered. The precision of our data does not allow us to detect the ionization of HEH_3 to HEH_2 (i.e., pK_1), and the pH dependence of activity is therefore best described by model 2b. Nonlinear regression of the data to the corresponding eq 2b indicates that, with CBZ-Cit-Arg-MCA, the Val133Ala/Ser205Glu enzyme is approximately 3 times more active when Glu205 is deprotonated. The pK_a's obtained from the profile with CBZ-Cit-Arg-MCA are in agreement with those obtained with the Phe and Arg derivatives, which is as expected since they reflect ionization of groups on the free enzyme and should therefore be independent of the substrate (Fersht, 1985).

DISCUSSION

The mutations of Val133 → Ala and Ser205 → Glu introduced into papain (Val133Ala/Ser205Glu) increase the activity of the enzyme toward CBZ-Arg-Arg-MCA by a factor of 22 while the activity with the CBZ-Phe-Arg-MCA substrate is 5-fold lower for this mutant as compared to wild type. The double mutant of papain therefore displays cathepsin B like specificity (see Table II). This change in specificity is also reflected in the pH dependence of activity toward hydrolysis of the MCA substrates by Val133Ala/Ser205Glu. The pH–activity profile for hydrolysis of CBZ-Arg-Arg-MCA is modulated in the acid limb by a group ionizing with a pK_a of 5.51 that does not affect the activity toward the substrate bearing a Phe in P_2, a situation very similar to that observed with cathepsin B (Khouri et al., 1991). The mutations therefore did produce the changes they were designed for, that is, to introduce cathepsin B specificity into papain. This was accomplished by replacing only two residues out of a total of 212 in papain while maintaining the overall activity of the enzyme.

Many attempts to modify the specificity of an enzyme have been made in the past. The change in specificity is generally coupled with a reduction of the catalytic activity of the enzyme. There are, however, few examples where an enzyme of altered specificity has been produced by site-directed mutagenesis while retaining its activity. Bone et al. (1989) have modified the substrate specificity of α-lytic protease with a concomitant increase in activity, but this was done at the expense of a broader specificity. Mutants of trypsin designed to modify the preference of this enzyme for basic amino acids have shown a high level of activity toward neutral substrates, but the change in specificity obtained was not the one desired (Graf et al., 1987). With subtilisin, however, Estell et al. (1986) and Wells et al. (1987a,b) have succeeded in modifying in a predictable way the specificity of subtilisin while maintaining or increasing the activity of the enzyme. In the present work, mutations were designed to reproduce in the plant protease papain the specificity for a basic arginine residue in the substrate's P_2 position characteristic of the mammalian protease cathepsin B. The desired effect has been achieved with little loss of activity toward the substrate CBZ-Phe-Arg-MCA and an important increase in activity toward CBZ-Arg-Arg-MCA with the Val133Ala/Ser205Glu mutant. This result is particularly surprising considering that only 29% of the residues in papain are identical in cathepsin B. To our knowledge, the closest example of such an exchange in specificity between two enzymes is given by the work of Wells et al. (1987a), where the substrate specificity of *Bacillus licheniformis* subtilisin has been introduced in *Bacillus amyloliquefaciens* subtilisin. However, these two enzymes show a 69% sequence homology and are obtained from two related bacterial species.

The results of this study also confirm the earlier suggestion that interaction between a Glu residue in the S_2 subsite of cathepsin B (Glu245 in cathepsin B numbering) and the basic side chain of an arginine in P_2 of a substrate is responsible for the affinity of cathepsin B toward substrates with basic residues in P_2 (Khouri et al., 1991). Computer modeling of the S_2 subsite of the mutant Val133Ala/Val157Gly/Ser205Glu, which mimics the S_2 subsite of cathepsin B, has been done by using the crystal structure determined by Drenth et al. (1976) for papain complexed with a chloromethyl ketone inhibitor (Figure 1). The resulting structure shows that the side chain of an Arg residue in position P_2 of a substrate can come within hydrogen-bonding distance of the glutamate residue located in the back of the S_2 cavity. The best evidence for the existence of such an interaction with the papain mutants is derived from the observation that the pH–activity profile for Val133Ala/Ser205Glu with CBZ-Arg-Arg-MCA displays a pK_a of 5.51 in the acid limb, a value very close to that of 5.46 attributed to ionization of Glu245 in cathepsin B (determined in presence of 5% acetonitrile). The activity of wild-type papain toward the substrates used in the present study is not influenced by a group with pK_a of ca. 5.5. With the CBZ-Phe-Arg-MCA substrate, no change in activity is observed upon ionization of Glu205 in Val133Ala/Ser205Glu, indicating that the side chain of the Phe residue does not interact significantly with Glu205 located in the back of the S_2 subsite.

The results with the citrulline derivative are also very useful in evaluating the contribution of electrostatic and hydrophobic interactions in the S_2 subsite to catalysis. Once again, the existence of an interaction between the citrulline side chain and Glu205 at the back of the S_2 subsite is best illustrated by the pH–activity profile with the Val133Ala/Ser205Glu mutant. The activity of the enzyme toward CBZ-Cit-Arg-MCA increases from $(k_{ca}/K_M)_1^{lim} = 1.86 \times 10^3$ M^{-1} s^{-1} to $(k_{cat}/K_M)_2^{lim} = 5.42 \times 10^3$ M^{-1} s^{-1} when Glu205 ionizes with a pK_a of 5.59 (see model 2b and Table III). From those numbers, the $\Delta\Delta G$ for the variation in enzyme–transition state binding energy upon ionization of the Glu205 residue can be estimated from the relation

$$\Delta\Delta G = -RT \ln\left[\frac{(k_{cat}/K_M)_2^{lim}}{(k_{cat}/K_M)_1^{lim}}\right] \qquad (3)$$

From the data presented in Table III we obtain $\Delta\Delta G = -0.63$ kcal/mol. This number compares well to the value of −0.48 kcal/mol obtained in the study with cathepsin B and reflects the difference in energy between a hydrogen bond (Cit···neutral Glu205 side chain) and a charged hydrogen bond (Cit···negatively charged Glu205 side chain) (Khouri et al., 1991). The kinetic parameters for hydrolysis of CBZ-Cit-Arg-MCA by Val133Ala/Ser205Glu papain are not too different from those of the wild-type enzyme, which suggests that the favorable effect of introducing a Cit–Glu205 interaction is mostly canceled by the unfavorable effect of removing the Val133 side chain. This can be taken as evidence for the contribution of hydrophobic contacts between the Cit side chain and Val133 in papain. For a substrate with a Phe in the P_2 position, the existence of a contact between the Val133 side chain and the phenyl ring of the substrate is most probable on the basis of the crystal structures of papain–chloromethyl ketone complexes, as illustrated in Figure 1a (Drenth et al., 1976). Accordingly, replacement of Val133 by Ala in the Val133Ala/Ser205Glu mutant causes a 5-fold decrease in k_{cat}/K_M with CBZ-Phe-Arg-MCA.

At low pH where the side chain of Glu205 is in its neutral protonated form, the activity is appreciable with the CBZ-Cit-Arg-MCA substrate [$(k_{cat}/K_M)_1^{lim} = 1.86 \times 10^3$ M^{-1} s^{-1}], while no detectable activity could be observed with the arginine derivative. The very low activity toward the CBZ-Arg-Arg-MCA substrate when the Glu205 side chain is not ionized has been noted before with cathepsin B (Polgâr & Csoma, 1987; Khouri et al., 1991). Polgâr and Csoma (1987) suggested that this was due to the presence of more than one charged residue in the S_2 subsite of cathepsin B interacting with the arginine side chain of the substrate. The residues suggested to be involved are Glu131, Arg202, and Glu245 (cathepsin B numbering). Through the deprotonation of the two Glu residue side chains at approximately pH 5.5, the charge in the neighborhood of the arginine side chain would change from positive at pH lower than 5.5 to negative at pH higher than 5.5. This would result in a stronger interaction of the substrate Arg side chain with the subsite and hence could explain the increase in activity observed. However, no charged residues (other than the engineered Glu205) are present in the S_2 subsite of papain and therefore, another explanation must be sought to explain this behavior. Of possible importance is the difference in solvation energy between the charged arginine and neutral citrulline side chains. Bringing the positively charged Arg side chain into the hydrophobic cavity of papain must be extremely costly in terms of the energy required to desolvate the side chain. However, at high pH (greater than 5.5) Glu205 is ionized and the enzyme can compensate for this energy loss by forming a favorable electrostatic interaction between the Arg and Glu side chains. This increase in interaction energy would be reflected in a higher level of activity, which could explain the observed pH effect.

From the data presented in Table II it is obvious that Val157 has an important role in the catalytic activity of papain. The coupling of binding interactions in the S_2 subsite of papain to catalytic reactivity of the enzyme has been suggested previously (Lowe & Yuthavong, 1971; Brocklehurst et al., 1979; Patel & Brocklehurst, 1982; Kowlessur et al., 1989). Crystal structures of papain inhibited with peptidyl chloromethyl ketones containing a phenylalanine in the P_2 position show that residues Val133 and Val157 make the most intimate contacts with the Phe side chain (Drenth et al., 1976). The mechanism by which these contacts can be used to increase the activity is not clear. The proximity of Val157 to His159 in the sequence of papain could be of importance in that aspect, since interaction of the substrate with Val157 might lead to conformational changes in the His159 region of the enzyme with a concomitant effect on activity.

It is difficult to dissect out the effect of the mutations on the three catalytic steps of the reaction, i.e., binding, acylation, and deacylation, since the rate-limiting step for reaction of the MCA substrates with papain (and its mutants) is not known. Unpublished results from our group suggest that, with CBZ-Phe-Arg-MCA, deacylation is rate-limiting and k_{cat} therefore corresponds to the rate of deacylation. The important decrease in k_{cat}/K_M observed between papain or Val133Ala/Ser205Glu and the triple mutant Val133Ala/Val157Gly/Ser205Glu is largely due to an effect on k_{cat}. This could be attributed to a decrease in the rate of deacylation, but it could also reflect a decrease in the rate of acylation with a change in the rate-limiting step with the CBZ-Phe-Arg-MCA substrate.

We have shown using site-directed mutagenesis that the specificity of a mammalian cathepsin B can be introduced in a plant protease by replacing only two amino acid residues in papain. This result supports the view that cathepsin B and papain possess a similar three-dimensional structure. It also confirms that combination of the information from crystal structures of papain and papain-inhibitor complexes with information from sequence alignment of related cysteine proteases constitutes a valid tool for the investigation of enzyme mechanisms and the design of enzymes with modified properties.

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Opposite Facial Specificity for Two Hydroquinone Epoxidases: (3-*si*,4-*re*)-2,5-Dihydroxyacetanilide Epoxidase from *Streptomyces* LL-C10037 and (3-*re*,4-*si*)-2,5-Dihydroxyacetanilide Epoxidase from *Streptomyces* MPP 3051†

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ABSTRACT: (3-*si*,4-*re*)-2,5-Dihydroxyacetanilide epoxidase (DHAE I), a key enzyme in the biosynthesis of the epoxysemiquinone antibiotic LL-C10037α by *Streptomyces* LL-C10037 [Gould, S. J., & Shen, B. (1991) *J. Am. Chem. Soc. 113*, 684–686], and (3-*re*,4-*si*)-2,5-dihydroxyacetanilide epoxidase (DHAE II) isolated from *Streptomyces* MPP 3051—which yields the (3*R*,4*S*)-epoxyquinone mirror image product of DHAE I—are described. DHAE I was purified 640-fold. Gel permeation chromatography indicated an M_r of 117 000 ± 10 000; SDS–PAGE gave a major band of 22 300 daltons, indicating that DHAE I is either a pentamer or hexamer in solution. The enzyme had a pH optimum of 6.5, a K_m of 8.4 ± 0.5 μM, and a V_{max} of 3.7 ± 0.2 μmol min^{-1} mg^{-1}. DHAE II was purified 1489-fold. The enzyme was shown to be a dimer of M_r 33 000 ± 2000, with 16 000-dalton subunits, with a pH optimum of 5.5 and a K_m of 7.2 ± 0.4 μM. Both enzymes required only O_2 and substrate; flavin and nicotinamide coenzymes had little or no effect. Neither catalase nor EDTA affected the activity of either enzyme, but complete inhibition of both was obtained with 1,10-phenanthroline. The activity of the purified DHAE I could be enhanced, but only by Mn^{2+} (relative V = 246 at 0.04 mM), Ni^{2+} (relative V = 266 at 0.2 mM), or Co^{2+} (relative V = 498 at 0.2 mM). Reconstitution from a DHAE I apoenzyme, generated by treatment with 1,10-phenanthroline followed by Sephadex G-25 chromatography, occurred only by addition of one of these three metals. It is proposed that DHAE I and DHAE II, and two other enzymes discussed, represent a hitherto unrecognized class of enzymes that should be called "hydroquinone monooxygenase (epoxidizing)".

Antibiotic LL-C10037α, produced by *Streptomyces* LL-C10037 (Lee et al., 1984) has structure **1** (Shen et al., 1990). Whole cell and cell-free studies show it is derived from the shikimate pathway via 3-hydroxyanthranilic acid, **2** (Whittle & Gould, 1987; Gould et al., 1989; Gould & Shen, 1991). As shown in Scheme I, 2,5-dihydroxyacetanilide, **3**, undergoes epoxidation catalyzed by (3-*si*,4-*re*)-2,5-dihydroxyacetanilide epoxidase (DHAE I)[1] to form epoxyquinone **4**. The desacetyl

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