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A general framework of cysteine-proteinase mechanism deduced from studies on enzymes with structurally different analogous catalytic-site residues Asp-158 and -161 (papain and actinidin), Gly-196 (cathepsin B) and Asn-165 (cathepsin H)

Kinetic studies up to pH 8 of the hydrolysis of *N*-α-benzyloxycarbonyl-L-arginyl-L-arginine 2-naphthylamide catalysed by cathepsin B and of L-arginine 2-naphthylamide catalysed by cathepsin H

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1. The pH-dependences of $k_{cat.}$, K_m and $k_{cat.}/K_m$ for the hydrolysis at 25°C at *I* 0.1 of L-arginine 2-naphthylamide catalysed by cathepsin H from bovine spleen were determined in the pH range approx. 4–8. 2. The pH-dependences of these kinetic parameters were determined also for the hydrolysis at 25°C at *I* 0.1 of *N*-α-benzyloxycarbonyl-L-arginyl-L-arginine 2-naphthylamide catalysed by cathepsin B (EC 3.4.22.1) from bovine spleen in the pH range 7–8, which extends the studies in acidic media reported by Willenbrock & Brocklehurst [(1984) *Biochem. J.* **222**, 805–814]. 3. These results are discussed and related to those from the reactivity-probe kinetics reported in the preceding paper [Willenbrock & Brocklehurst (1985) *Biochem. J.* **227**, 511–519] and to known structural features present in rat liver cathepsins B and H and in papain (EC 3.4.22.2) and actinidin (EC 3.4.22.14). 4. Consideration of the kinetic data leads to the suggestion that in the cysteine proteinases rearrangement of intimate S^-/ImH^+ ion-pairs in catalytic sites is brought about by a combination of field effects in the immediate vicinity of the ion-pair and consequences of protonic dissociation of a group with pK_a 5–6 remote from the catalytic site. 5. The contributions of the two types of effect seem to differ from enzyme to enzyme. Of the four cysteine proteinases considered, only cathepsin B exerts an absolute requirement for the proton-deficient form of a group with pK_a 5–6 for catalytic activity. Protonic dissociation with pK_a 5–6 enhances catalytic activity in cathepsin H and in actinidin and appears to have little or no effect in papain. Only cathepsin B lacks a polar or negatively charged side chain in the residue analogous to Asp-158 in papain, and this is suggested to account for its total dependence on a protonic dissociation remote from the catalytic site.

Evidence is accumulating that the cysteine proteinases may constitute a particularly valuable series of enzymes with which to explore the possibility of using natural structural variation in analogous or homologous enzymes as a tool in the study of catalytic mechanism (see Brocklehurst *et al.*, 1983; Salih & Brocklehurst, 1983; Brocklehurst *et al.*, 1984; Willenbrock & Brocklehurst, 1984). It became evident that the two most abundant cysteine proteinases present in animal tissues, cathepsin B (EC 3.4.22.1) and cathepsin H, are valuable targets for mechanistic study when Takio *et al.* (1983) reported the complete amino acid sequences of both enzymes from rat liver. Comparison of the amino acid sequences of cathepsins B and H with each other and with those of papain (EC 3.4.22.2) (Husain & Lowe, 1968*a*,*b*) and of actinidin (EC 3.4.22.14) (Carne & Moore, 1978) demonstrated a striking homology in the primary struc-

Abbreviations used. Z-, benzoyloxycarbonyl; -NNap, 2-naphthylamide.

tures of these enzymes, but with important differences. Overall, the sequence of rat liver cathepsin H corresponds more closely to the sequences of the plant enzymes papain and actinidin than to that of rat liver cathepsin B, and Takio *et al.* (1983) suggested that cathepsin B might have diverged from the common ancestral gene long before cathepsin H. The sequences close (say within ten residues) on the *N*-terminal side to the catalytic-site cysteine residue (residue 25 in papain and in actinidin, residue 29 in cathepsin B and residue 26 in cathepsin H) are remarkably similar in all four enzymes, with a particularly close correspondence between these sequences in papain and cathepsin H. The limited sequence data available for bovine spleen cathepsin B (Pohl *et al.*, 1982) shows that this enzyme and the corresponding rat liver enzyme also are closely similar in this respect. The sequences around the catalytic-site histidine residue (indicated below in all cases by an asterisk) have a lower degree of identity, although some homology in this region also is apparent, particularly between cathepsin H and papain. Of particular interest from a mechanistic viewpoint is the fact that the residue adjacent on the *N*-terminal side to the catalytic-site histidine residue is not aspartic acid in either cathepsin B or cathepsin H, whereas it is well known that this residue is aspartic acid in papain (158) and in actinidin (161). Short sequences around the catalytic-site histidine residues (197 in cathepsin B, 166 in cathepsin H, 162 in actinidin and 159 in papain) with residues homologous with those in papain underlined are:

for papain, Val-Asp-His*-Ala-Val-Ala-Ala;
for actinidin, Val-Asp-His*-Ala-Ile-Val-Ile;
for cathepsin H, Val-Asn-His*-Ala-Val-Leu-Ala;
for cathepsin B, Gly-Gly-His*-Ala-Ile-Arg-Ile.

Of particular note is the fact that the residues equivalent to Asp-158 in papain and Asp-161 in actinidin are glycine and asparagine in cathepsins B and H respectively. Because mechanistic roles for the side chain of Asp-158 in papain have been the subject of considerable discussion (see, e.g., Brocklehurst *et al.*, 1981) this residue is a prime candidate for structural change in papain by site-directed mutagenesis. The two most obvious changes to make are replacement of Asp-158 in one case by an asparagine residue and in the other by a glycine residue. The former preserves the possibility of contacts by hydrogen-bonding while eliminating the negative charge, and the latter eliminates all possibilities of significant contacts either with other catalytic-site components or with substrate or reactivity probe. It is a matter of considerable interest, therefore, that these two structural changes are provided naturally in the two cathepsins, $-CH_2-CO_2^- \rightarrow -CH_2-CONH_2$ in cathepsin H and $-CH_2-CO_2^- \rightarrow -H$ in cathepsin B.

In a previous paper (Willenbrock & Brocklehurst, 1984) we reported considerable differences between cathepsin B and papain deduced by using a combination of substrate-catalysis kinetics and two-protonic-state reactivity-probe kinetics, all in acidic media. That paper showed also that cathepsin B from bovine spleen and cathepsin B from rat liver behave in a closely similar manner with regard to both catalytic and thiol reactivity characteristics. This is a useful observation, because it allows the sequence data obtained by Takio *et al.* (1983) for the rat liver enzyme to be used, albeit provisionally, in the interpretation of results obtained with bovine spleen enzyme, which can be obtained more easily in larger quantities. It has not been possible so far for us to obtain sufficient quantities of rat liver cathepsin H to demonstrate analogous close similarity with the bovine spleen enzyme. In view of the findings with cathepsin B from the two sources, however, we have tentatively assumed similarity also between cathepsin H from the two sources and have commented briefly on our kinetic data obtained with the bovine spleen enzyme in relation to the sequence data that are available for the rat liver enzyme.

In the accompanying paper (Willenbrock & Brocklehurst, 1985), the catalytic-site characteristics of cathepsins B and H, papain and actinidin were compared by using data from reactivity-probe kinetics. The present paper reports the results of a kinetic study of substrate hydrolysis catalysed by cathepsin H over a wide range of pH up to pH 8 and extension of an analogous study on cathepsin B up to pH 8. This is not a trivial extension, because substantial changes in the kinetic constants occur in the pH range 7–8. It proved possible to carry out reliable kinetic studies on these enzymes up to pH 8, despite their well-known instability outside acidic media, by using continuous spectroscopic assays and by working at a lower temperature (25°C) than usually had been used hitherto. The considerable differences between cathepsin B, cathepsin H, papain and actinidin detected both in the present work and in that in the accompanying paper (Willenbrock & Brocklehurst, 1985) are discussed, and a general framework within which variation in cysteine-proteinase characteristics may be further understood is proposed.

Materials and methods

The materials and many of the methods have been described previously (see Willenbrock & Brocklehurst, 1984, 1985). These include isolation of the enzymes from bovine spleen, buffers, kinetic methods, data processing and spectroscopic properties of cathepsins B and H and of 2-naphthyl-

amine. Catalytic activities were determined by the continuous spectroscopic assays, with Z-Arg-Arg-NNap for cathepsin B and Arg-NNap for cathepsin H, as described by Willenbrock & Brocklehurst (1984). Most of the catalytic data were obtained by the half-time method of Wharton & Szawelski (1982) after establishing the adherence of the reactions to the Michaelis–Menten equation by conventional initial-rate studies at selected pH values. Values of $k_{cat.}$ and K_m obtained by both methods at these pH values were closely similar ($\pm < 10\%$). By studying substrate hydrolysis at 25°C and using a continuous assay, it was possible to study the kinetics of catalysis up to pH 8.0.

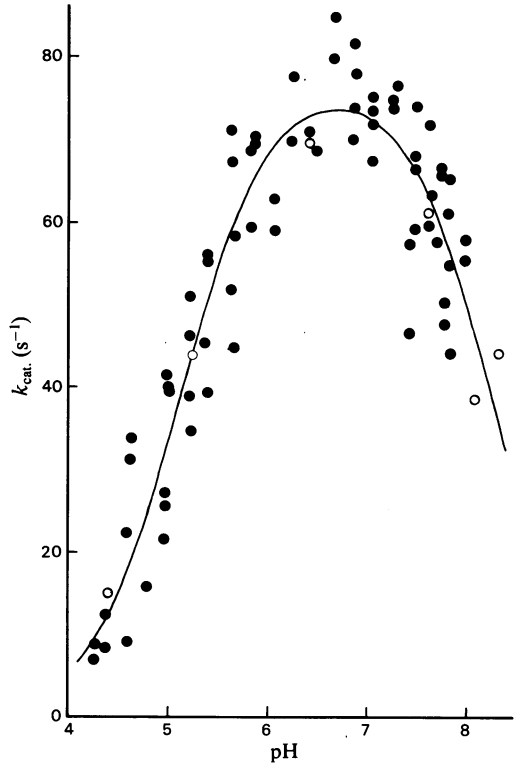


Fig. 1. *pH-dependence of $k_{cat.}$ for the hydrolysis of Z-Arg-Arg-NNap catalysed by cathepsin B at 25°C at 10.1*
The points are experimental [○, from initial-rate measurements at different substrate concentrations; ●, determined by the half-time method of Wharton & Szawelski (1982)] and the line corresponds to:

$$k_{cat.} = \frac{\tilde{k}_{cat.}}{1 + \frac{[H^+]}{K_{II}} + \frac{K_{II}}{[H^+]}}$$

where $\tilde{k}_{cat.} = 77\,s^{-1}$, $pK_I = 5.1$ and $pK_{II} = 8.3$. The data at pH values $\leqslant 7$ are taken from Willenbrock & Brocklehurst (1984).

Results and discussion

pH-dependence of the kinetics of substrate hydrolysis catalysed by cathepsins B and H

The pH-dependences in the pH range approx. 4–8 of $k_{cat.}$, K_m and $k_{cat.}/K_m$ for the cathepsin B-catalysed hydrolysis of Z-Arg-Arg-NNap are shown in Figs. 1–3 respectively, and the analogous pH-dependences for the cathepsin H-catalysed hydrolysis of Arg-NNap are shown in Figs. 4–6. It seems useful to show the pH-dependences of K_m (Figs. 2 and 5) despite their complex significance (see, e.g., Brocklehurst & Dixon, 1976), because the decrease in K_m in alkaline media for the cathepsin B reaction, which contrasts with the increase in K_m in alkaline media for the cathepsin H reaction, is clearly responsible for the unusual form of the pH-dependence of $k_{cat.}/K_m$ shown in Fig. 3. Our earlier report on the cathepsin B reaction in acidic media (Willenbrock & Brocklehurst, 1984) showed both $k_{cat.}$ and $k_{cat.}/K_m$ to increase with increasing pH

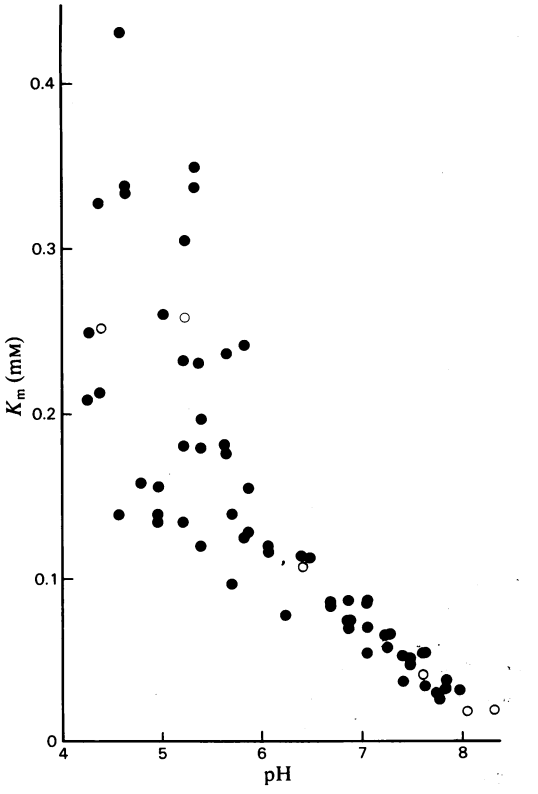


Fig. 2. *pH-dependence of K_m for the hydrolysis of Z-Arg-Arg-NNap catalysed by cathepsin B at 25°C at 10.1*
The data points (○ and ●) were obtained as indicated in Fig. 1 legend. Because of the complex significance of the pH-dependence of K_m, a theoretical line with characterizing parameters is not given.

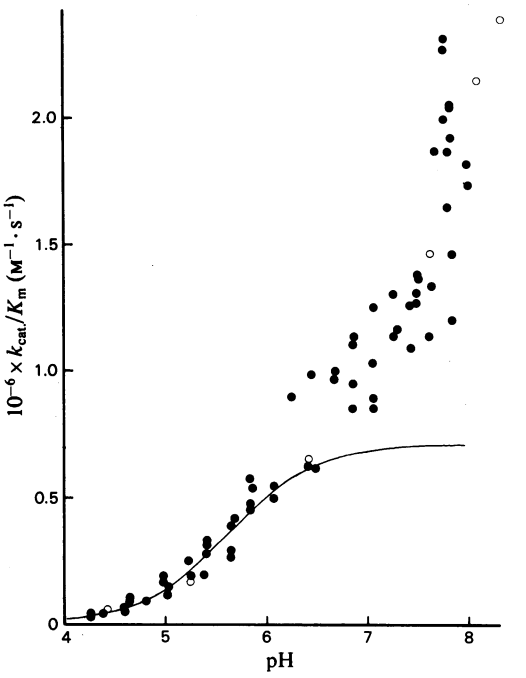


Fig. 3. *pH-dependence of $k_{cat.}/K_m$ for the hydrolysis of Z-Arg-Arg-NNap catalysed by cathepsin B at 25°C at I 0.1*
The data points (○ and ●) were obtained as indicated in Fig. 1 legend. The line corresponds to:

$$\frac{k_{cat.}}{K_m} = \frac{\tilde{k}_{cat.}/\tilde{K}_m}{1 + \frac{[H^+]}{K_a}}$$

where $\tilde{k}_{cat.}/\tilde{K}_m = 7.1 \times 10^5 M^{-1} \cdot s^{-1}$ and $pK_a = 5.6$ and is taken from Willenbrock & Brocklehurst (1984).

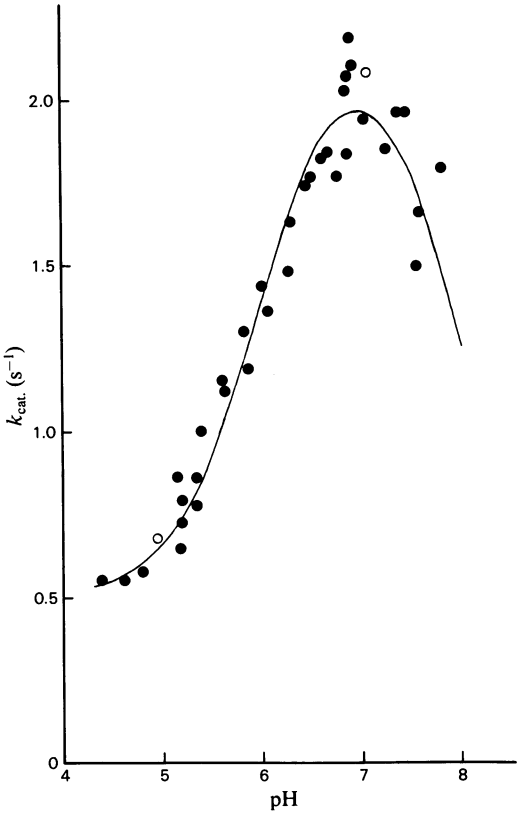


Fig. 4. *pH-dependence of $K_{cat.}$ for the hydrolysis of Arg-NNap catalysed by cathepsin H at 25°C at I 0.1*
The data points (○ and ●) were obtained as indicated in Fig. 1 legend. The line corresponds to:

$$k_{cat.} = \frac{\tilde{k}_{cat.1}}{1 + \frac{[H^+]}{K_I} + \frac{K_{II}}{[H^+]}} + \frac{\tilde{k}_{cat.2}}{1 + \frac{[H^+]}{K_{II}} + \frac{K_{III}}{[H^+]}}$$

where $\tilde{k}_{cat.1} = 0.5 s^{-1}$, $\tilde{k}_{cat.2} = 2.3 s^{-1}$, $pK_I < 4$, $pK_{II} = 6.0$ and $pK_{III} = 8.1$. The value of $\tilde{k}_{cat.1}$ was estimated by eye and pK_I was set at a low value so that it did not affect the computer optimization used to obtain the values of the other characterizing parameters listed above.

across pK_a values 5–6. The results of the extended study (Figs. 1 and 3) now show (*a*) that the variation in $k_{cat.}$ with pH takes the form of a bell-shaped curve with pK_I 5.1 and pK_{II} 8.3 and (*b*) that the sigmoidal increase in $k_{cat.}/K_m$ with increasing pH in acidic media is augmented by an even greater increase in weakly alkaline media. The corresponding pH-dependences for the cathepsin H reaction are somewhat different. Thus the pH-dependence of $k_{cat.}/K_m$ (Fig. 6) is bell-shaped, because the decrease in $k_{cat.}$ in alkaline media (Fig. 4) is augmented by the change in K_m (Fig. 5) and not offset by it as in the case of the cathepsin B reaction. Of particular note is the additional reactive state of the enzyme–substrate complex produced by protonic dissociation with $pK_a < 4$ displayed by the plateau value of $k_{cat.}$ in acidic media (Fig. 4). The value of $k_{cat.}$ for reaction in this plateau region is approx. 23% of the value of $k_{cat.}$ for the reaction in the bell-shaped component at higher pH values. There is no evidence for a corresponding plateau region in the pH–$k_{cat.}$ profile for the cathepsin B reaction. The data in Fig. 1 show that, if such a plateau exists, its reactivity must be less than 6% of the reactivity of the protonic state in the bell-shaped component around pH 6.5.

Mechanistic implications of the combined kinetic studies of catalysis and of covalent modification of the catalytic-site thiol group

The conclusion from experiments on cathepsin

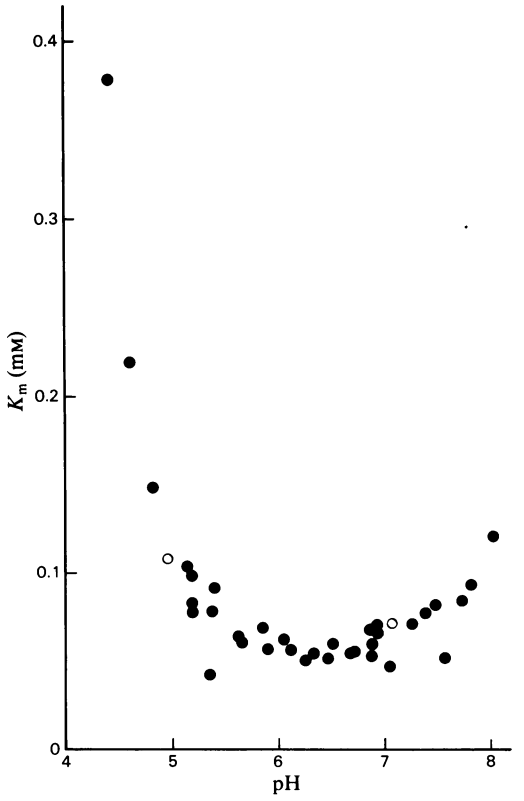


Fig. 5. *pH-dependence of K_m for the hydrolysis of Arg-NNap catalysed by cathepsin H at 25°C at I0.1*
The data points (○ and ●) were obtained as indicated in Fig. 1 legend. The comment about the lack of a theoretical line given in Fig. 2 legend applies here also.

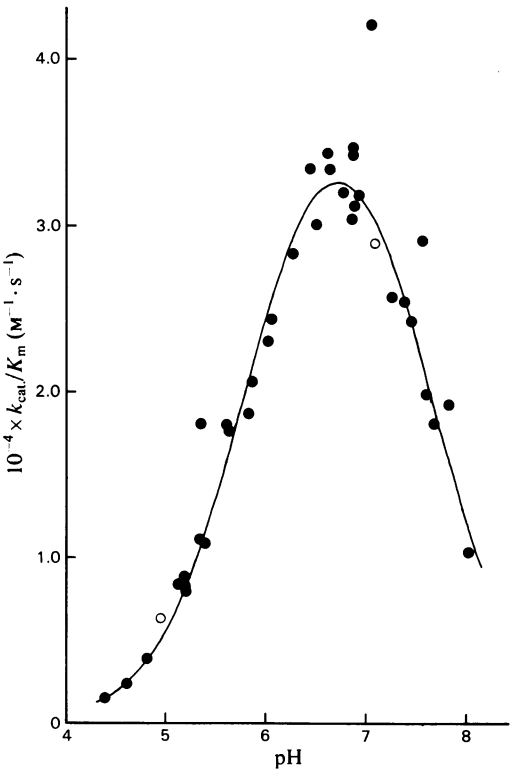


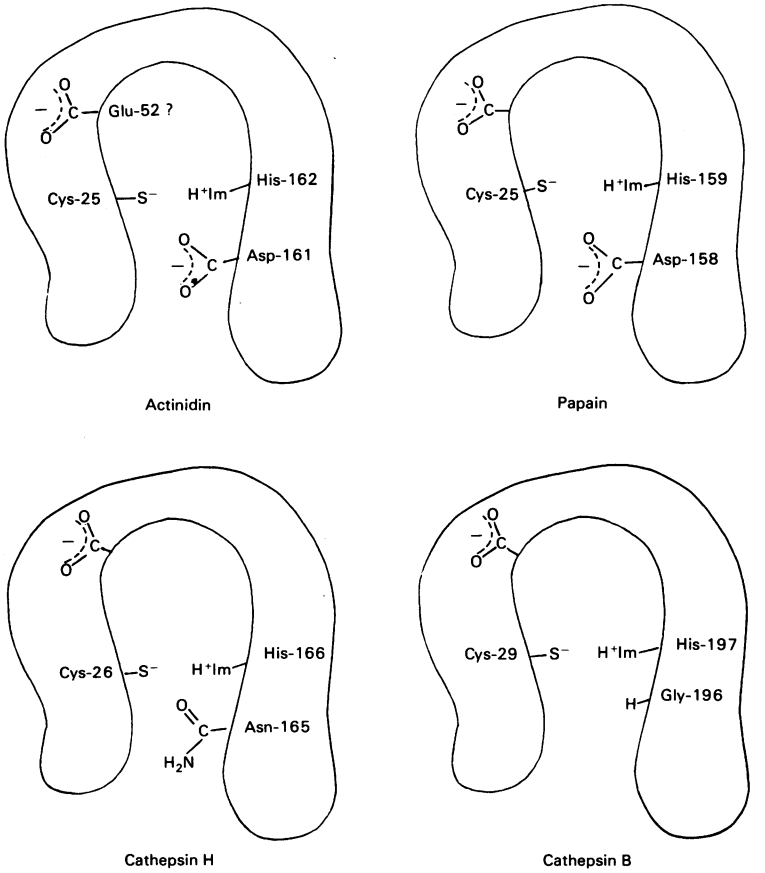
Fig. 6. *pH-dependence of $k_{cat.}/K_m$ for the hydrolysis of Arg-NNap catalysed by cathepsin H at 25°C at I0.1*
The data points (○ and ●) were obtained as indicated in Fig. 1 legend. The line corresponds to an equation of the same form as that given in Fig. 1 legend, where $\tilde{k}_{cat.}/\tilde{K}_m = 4.1 \times 10^{-4} M^{-1} \cdot s^{-1}$, $pK_I = 5.8$ and $pK_{II} = 7.6$.

B in acidic media that S^-/ImH^+ ion-pair is produced by protonic dissociation associated with a molecular pK_a of 3.4 but is not able to become acylated (on S) by substrate until a further protonic dissociation (pK_a 5.6 in the free enzyme and pK_a 5.1 in the enzyme–substrate complex) has occurred was discussed by Willenbrock & Brocklehurst (1984). From the present work it is possible to add the conclusion, from the pH-dependence of $k_{cat.}$, that acid catalysis (presumably by ImH^+ of His-197; pK_a 8.3 in the enzyme–substrate complex) is required to complete the acylation process.

In the case of cathepsin H, an interactive system (again presumably containing the S^-/ImH^+ ion-pair) was shown by reactivity-probe kinetics (Willenbrock & Brocklehurst, 1985) to be generated by protonic dissociation with molecular pK_a 2.7. The pH-dependence of $k_{cat.}$, which probably reflects the formation of acyl-enzyme from adsorptive complex devoid of complications arising from the pH-dependence of K_m, suggests a significant difference from the acylation of cathepsin B. In the acylation of cathepsin B the further event associated with protonic dissociation of pK_a 5–6 appears to be obligatory. For cathepsin H, acylation of the ion-pair form generated by protonic dissociation with $pK_a < 4$ (presumably pK_a 2.7) proceeds at an appreciable rate (Fig. 4) although the acylation is considerably enhanced (about 5-fold) by protonic dissociation with pK_a 6. Thus acylation of cathepsin H appears to represent a situation intermediate between the acylation of cathepsin B, where proton loss with pK_a 5–6 from the enzyme form containing the S^-/ImH^+ ion-pair is obligatory, and the acylation of papain, where reactivity is fully expressed in the enzyme form in which two co-operatively linked protonic dissociations of pK_a 3–4 have occurred [see Willenbrock & Brocklehurst (1984) for a discussion of the papain case]. The pK_a of 5–6 in papain detected by the 2,2′-dipyridyl disulphide reactivity probe appears to have a negligible influence on papain's catalytic activity.

Towards a general framework of cysteine-proteinase structure and mechanism

The results presented above and in the accompanying paper (Willenbrock & Brocklehurst, 1985) suggest the basis for a general framework within which the cysteine-proteinase mechanism might begin to be more clearly understood by taking account of the influence of an additional ionizing group remote from the catalytic site. This is illustrated schematically in Scheme 1, which shows simple representations of assemblies of four functional groups within each of the cysteine proteinases papain, actinidin and cathepsins B and H. An interactive system involving the side chains of a cysteine residue and a histidine residue is well established as a common feature of cysteine-proteinase catalytic sites, and S^-/ImH^+ ion-pair is a plausible component of such systems. If such ion-pairs are mutually solvated (intimate ion-pairs), an important question is how, in the acylation process, do the ion-pair components separate from each other and take up positions that permit performance of their respective roles as nucleophile and general acid. It now seems possible to suggest that this rearrangement of structure is brought about by a combination of (I) field effects in the immediate vicinity of the S^-/ImH^+ ion-pair and (II) consequences of protonic dissociation of a group with pK_a 5–6 remote from the catalytic site. The contributions of effects (I) and (II) seem to differ from enzyme to enzyme, and it is possible or even probable that one or both of these effects might be modulated by substrate or reagent binding. For example, the association of the 2-pyridyl nitrogen atom of unsymmetrical disulphide probes containing this group in one half of the molecule with a catalytic-site proton donor (probably ImH^+ of His-159) appears to be regulated by binding interactions in or near the S_2-subsite of papain



Scheme 1. *Schematic representations of four cysteine proteinases showing in each case three catalytic-site residues and the carboxylate anion (not numbered except for actinidin) postulated to be in a hydrophobic environment remote from the catalytic site*
In actinidin a possible site for this carboxylate anion might be the side chain of Glu-52 (see the text).

(Brocklehurst & Malthouse, 1980) and possibly also in other regions of the binding site (G. Patel & K. Brocklehurst, unpublished work).

The pK_a of the group remote from the catalytic site suggests that it could be a carboxy group in a hydrophobic environment. Ionization of such a group in the hydrophobic cleft, which is clearly a feature of the structures of papain and actinidin, could well promote movement in the cleft involving decoupling of S^- and ImH^+, which are on opposite walls (at least in papain and actinidin). Some possible consequences of this type of movement in papain have been discussed by Lowe & Yuthavong (1971) and by Drenth *et al.* (1975).

A tentative identification of the group with pK_a 5–6 is described in terms of Baker's (1980) actinidin structure. This enzyme, like papain, has a double domain structure: domain I (residues 19–115 and 214–218) includes the catalytic-site thiol group (Cys-25) and domain II (residues 1–18 and 116–213) includes the catalytic-site carboxamide–imidazole–indole system (Asn-182, His-162 and Trp-184) and the carboxy group of Asp-161. Actinidin contains three buried carboxy groups, those of Glu-35, Glu-50 and Glu-52. Glu-35, Glu-50 and Lys-181 all lie within the interface between domains I and II, along with Lys-17, partly buried at the end of the interface, and beyond it Glu-86. Although both domains have a congregation of non-polar side chains in their interior, the interface between them is made up largely of polar side chains and a network of buried water molecules. The charge on two of the buried carboxylate groups, Glu-35 and Glu-50, is partly neutralized by Lys-17 and further by interaction with Lys-181, which is itself linked to Glu-35 through two water molecules. The other buried carboxy group, that of Glu-52, is of particular interest as a possible candidate for the group with pK_a 5–6. It lies in a partly hydrophobic environment adjacent to the side chains of the Ile-83 and Phe-76. One carboxy oxygen atom receives a hydrogen bond from the main-chain amido group of residue 84 (Asn), and the other, in the crystal, is 0.266nm from a buried solvent molecule or ion, possibly an NH_4^+ ion. Differences in the molecular micro-environments in other cysteine proteinases of carboxy groups analogous to Glu-52 in actinidin, and in the intervening structure linking this region to the catalytic site, might account for the variations in the kinetic effects produced by ionization of the group with pK_a 5–6 discussed above.

The four cysteine proteinases considered in Scheme 1 appear to constitute a series in which the contributions of effects I and II differ considerably. In papain the field effect, possibly associated in some way with Asp-158, appears to be wholly responsible for controlling both catalytic activity and reactivity of Cys-25 towards most reactivity probes (see Brocklehurst *et al.*, 1984, and references cited therein), although evidence for some influence of the group with pK_a 5–6 is apparent in the reactivity of papain towards 2,2′-dipyridyl disulphide monocation (Shipton & Brocklehurst, 1978). It remains a matter of concern that actinidin, which, according to the crystallographic data, has a catalytic site (with the common $S^-/ImH^+/CO_2^-$ system) closely similar to that of papain, displays considerably different catalytic-site properties (see, e.g., Brocklehurst *et al.*, 1983; Salih & Brocklehurst, 1983; Brocklehurst *et al.*, 1984). It is interesting that, in marked contrast with the situation found with papain, ionization of the group of pK_a 5–6 has a considerable effect both on the reactivity of the catalytic-site thiol group of actinidin towards benzofuroxan (Salih & Brocklehurst, 1983) and on $k_{cat.}/K_m$ for substrate hydrolysis (E. Salih, M. Jarvis & K. Brocklehurst, unpublished work). Structural differences remote from the immediate vicinity of the catalytic site in actinidin appear to dictate that effect II outweighs effect I, despite the presence of a carboxylate anion analogous to Asp-158 in papain. The group of pK_a 5–6 clearly affects the catalytic activity of cathepsin H, although a requirement for its existence in proton-deficient form is not absolute, as it is in cathepsin B. It may be that the polar carboxamide side chain of Asn-165 in cathepsin H can fulfil partially the role that might be played by Asp-158 in papain. The equivalent residue in cathepsin B (Gly-196) clearly can have no positive function, which could explain why the effect produced by the remote ionizable group is mandatory in this enzyme for catalytic competence.

In other cysteine proteinases these ionic and polar interactions may be further modulated if other ionic or polar groups are present in the catalytic site or other strategic regions of the protein. A dramatic example of this is found with the little-studied enzyme chymopapain A. On the basis of its remarkable lack of reactivity towards the 2,2′-dipyridyl disulphide monocation and its equally remarkable residual catalytic activity in strongly alkaline media, this enzyme is predicted to contain an additional (and high-pK_a) cationic side chain (presumably arginine or lysine), in or near to its catalytic site (B. S. Baines, E. Salih & K. Brocklehurst, unpublished work; Salih, 1984).

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