A marked gradation in active-centre properties in the cysteine proteinases revealed by neutral and anionic reactivity probes

Reactivity characteristics of the thiol groups of actinidin, ficin, papain and papaya peptidase A towards 4,4'-dipyridyl disulphide and 5,5'-dithiobis-(2-nitrobenzoate) dianion

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1. The kinetics of the reactions of the catalytic-site thiol groups of actinidin (the cysteine proteinase from *Actinidia chinensis*), ficin (EC 3.4.22.3), papain (EC 3.4.22.2) and papaya peptidase A (the other monothiol cysteine proteinase component of *Carica papaya*) with 4,4'-dipyridyl disulphide (4-Py-S-S-4-Py) and with 5,5'-dithiobis-(2-nitrobenzoate) dianion (Nbs_2^{2-}) were studied in the pH range approx. 6–10. These studies provided the pH-independent second-order rate constants ($\tilde{k}$) for the reactions of the two probe reagents with the catalytic-site thiolate anions each in the environment of a neutral histidine side chain where an active-centre carboxy group would be ionized. 2. The ratio $R = \tilde{k}_{\text{Nbs}_2^{2-}}/\tilde{k}_{4\text{-Py-S-S-4-Py}}$ provides an index of the catalytic-site solvation properties of the four cysteine proteinases and varies markedly from one enzyme to another, being 0.80 for papaya peptidase A (0.86 for the model thiol, 2-mercaptoethanol), 29 for actinidin, 0.18 for ficin and 0.015 for papain. These differences appear to derive mainly from the response of the enzyme to the negative charge on Nbs_2^{2-} . 3. Possible implications of these results for (a) mechanisms of cysteine proteinase catalysis and (b) the possibility of using series of functionally related enzymes in the study of mechanism are discussed.

To understand the molecular basis of the rate enhancements that are characteristic of enzyme catalysis, it is necessary to resolve the interaction energy between active centre and substrate into its components and to determine their relative importance. It has long been recognized that ionizable functional groups in enzymes play important roles in catalysis, and Warshel (see Warshel, 1981a, and references cited therein) has emphasized the probable importance of the role of enzyme charges and dipoles in stabilizing by solvation the ionic resonance forms of transition states. The enzyme catalytic site is viewed as a 'supersolvent' for a particular transition state, with enzyme dipoles pre-orientated to provide an electric field complementary to the charge distribution of the transition state (Warshel, 1978, 1981b). One component of this catalytically relevant electric field might be a piece of α -helix,

Abbreviations used: 4-Py-S-S-4-Py, 4,4'-dipyridyl disulphide; Nbs_2^{2-} , 5,5'-dithiobis-(2-nitrobenzoate) dianion.

which, in proteinases, is often found close to the active-centre region (Van Duijn *et al.*, 1980). Another possible candidate for a field component in the case of the cysteine proteinases is the active-centre carboxy group (aspartic acid-158 in papain), the role of which is yet to be established (see, e.g., Brocklehurst *et al.*, 1981a, 1982, and references cited therein).

One approach to the study of active-centre chemistry involves the use of site-specific reactivity probes. For those cysteine proteinases that contain only one (accessible) thiol group per molecule, specific reaction at the catalytic-site thiol group can be ensured at all pH values by using probe reagents containing electrophilic sulphur, such as disulphides (Brocklehurst, 1979). These reagents also fulfil the optimal requirement for mechanistic study of providing a single-step covalency change. The reactivity-probe technique is particularly relevant to the study of catalysis when, as is the case with the cysteine proteinases (see, e.g., Brocklehurst *et al.*, 1981a), the

enzyme nucleophile that reacts with the probe serves a nucleophilic purpose in the catalytic act itself. The influence of the microenvironment of the active centre on the interaction energy of a given nucleophile (active-centre)-electrophile (probe) pair can be assessed by changing the structure of the electrophilic reagent. In particular, by using a pair (or series) of reagents of similar size, each containing a similar electrophilic centre but with different ionic characteristics, the problem of isolation of solvation effects from at least gross steric effects can be obviated. The most important single impediment to mechanistic study of enzymes, which sets it apart from mechanistic study of simpler organic molecules, is the lack of opportunity, generally, to vary systematically the structure of the component of the reactant state that contains the nucleophile. In the case of an enzyme it is probable that a multiplicity of microenvironmental factors determines the reactivity characteristics of an active-centre nucleophile. In an attempt to ascertain whether a family of similar enzymes might provide an opportunity to study variation in active-centre structure, we have studied the properties of the active centres of four cysteine proteinases (each of which contains only one accessible thiol group per molecule) by using the neutral probe 4,4'-dipyridyl disulphide (4-Py-S-S-4-Py) and the anionic probe 5,5'-dithiobis-(2-nitrobenzoate) dianion (Nbs_2^{2-}). The present paper reports considerable differences among the properties of all four enzymes, ranging from actinidin [the cysteine proteinase from *Actinidia chinensis* (kiwifruit)] with a strong preference for the anionic probe, through papaya peptidase A [the other major monothiol cysteine proteinase component other than papain (EC 3.4.22.2) of *Carica papaya*] with little or no preference for anionic or neutral probe (as is the case with simple thiols in aqueous media), through ficin (EC 3.4.22.3), which repels the anionic probe, and further to papain, which repels the anionic probe even more markedly. The result suggests that the use of series of functionally related enzymes might well fill the gap in the techniques available for enzyme study referred to above, and may be useful in the delineation of the evolution of optimal catalytic devices. Possible implications of these results for mechanisms of catalysis by cysteine proteinases are discussed.

Materials and methods

Materials

The latex of *Carica papaya* from which papain and papaya peptidase A were prepared was the spray-dried refined product known to have been prepared by the Boudart (1969, 1970) process in Zaïre, and was kindly supplied by Powell and Scholefield, Liverpool, U.K.

The method of preparation of papain free from contamination by other enzymes was developed (Baines & Brocklehurst, 1979) for use with the Boudart latex. It is noteworthy that this type of latex is no longer generally available, and that when this method is applied to some of the types of papaya latex currently offered commercially considerable contamination of the papain product results. It may now be necessary, therefore, to develop isolation procedures for each particular type of latex that becomes available.

Fresh kiwifruit (Chinese gooseberries) were obtained from the New Covent Garden Market, London S.E.8, U.K. On delivery, the fruit was washed with 1 mM-EDTA solution and weighed into 1 kg lots (11–13 fruits). Each 1 kg lot was pulped by using a food mixer, and the pulp was stored in freezer bags in the deep-freeze until required.

The dried latex of *Ficus glabrata* was obtained from Hughes and Hughes (Romford, Essex, U.K.).

Enzymes

Active, activator-free, samples of the four cysteine proteinases were freshly prepared as reported previously: papain (Baines & Brocklehurst, 1979); ficin (Malthouse & Brocklehurst, 1976); papaya peptidase A (Baines & Brocklehurst, 1982); actinidin (Brocklehurst *et al.*, 1981*b*). In the preparation of actinidin, frozen fruit pulp (1 kg) was allowed to thaw at room temperature (approx. 22°C) and was then extracted with 500 ml of 20 mM-L-cysteine, pH 5.0, containing 1 mM-EDTA, by stirring the mixture for 1 h. In the original report of this procedure, the concentration of L-cysteine in the extraction medium was wrongly printed as 200 mM.

Concentrations of enzyme protein were calculated from measurements of A_{280} by using the following values of ϵ_{280} given in $\text{M}^{-1}\cdot\text{cm}^{-1}$: for papain, 5.6×10^4 (see Brocklehurst *et al.*, 1973); for ficin, 4.6×10^4 (Hollaway, 1967); for actinidin, 5.51×10^4 (see Brocklehurst *et al.*, 1979); for papaya peptidase A, 4.41×10^4 (see Baines & Brocklehurst, 1982).

Reactivity probes

Saturated solutions of 4-Py-S-S-4-Py (approx. 3.8 mM) were prepared by stirring the solid reagent in water at room temperature for 20 min, after which excess of solid was removed by filtration. The concentration of 4-Py-S-S-4-Py was determined spectroscopically at pH 7 by using $\epsilon_{247} = 1.63 \times 10^4 \text{ M}^{-1}\cdot\text{cm}^{-1}$ (Grassetti & Murray, 1967), and was checked occasionally by thiolysis with 2-mercaptoethanol (see Brocklehurst & Little, 1973). The concentration of the thiolysis product, pyridine-4-thione, was calculated from spectroscopic measurements and

$$\epsilon_{324} = 1.98 \times 10^4 / (1 + 2.51 \times 10^{-9} / [\text{H}^+]) \text{ M}^{-1}\cdot\text{cm}^{-1}$$

(see Grasseti & Murray, 1967; Brocklehurst & Little, 1973).

Solutions of Nbs_2^{2-} (approx. 3.95 mM) were prepared by adding small volumes (approx. 0.1 ml at a time) of 0.1 M-NaOH to a vigorously stirred suspension of the solid acid in water, the pH being maintained within the range 7.5–8.5 to prevent hydrolysis, followed by removal of undissolved solid by filtration. The concentration of Nbs_2^{2-} was determined as described for 4-Py-S-S-4-Py by using $\epsilon_{325} = 1.8 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Brocklehurst & Little, 1973) and $\epsilon_{412} = 1.36 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Ellman, 1959) for the chromophoric thiolysis product, 5-mercapto-2-nitrobenzoate anion.

Kinetics

Progress curves for the various stoichiometric thiol–disulphide interchanges were obtained at 25°C and at I 0.1 by using a Dionex model D110 stopped-flow system coupled to a Tektronix S1 03N storage oscilloscope and recorded with a Polaroid camera. Data from the stopped-flow records were analysed by using conventional first-order logarithmic plots. The concentrations in the mixing chamber of the stopped-flow apparatus were: enzyme, 3–10 μM ; probe reagent, 0.1–2.0 mM. Other materials and methods including buffers and determination of the characterizing parameters of the pH– k profiles by an optimization procedure are as reported previously (see Baines & Brocklehurst, 1982).

Results and discussion

Papain, actinidin, ficin and papaya peptidase A and the ionic components of their catalytic sites

Each of these four cysteine proteinases contains only one reactive thiol group per molecule. In each case this group, which is part of the catalytic site, forms part of an interactive system in which nucleophilic character in the thiol group is maintained in acidic media by its association with at least one other acid–base side chain. The presence of these interactive systems is revealed most easily by using thiol-specific two-protonic-state reactivity probes (Brocklehurst, 1982). The presence and ionization characteristics of the interactive system have been reported for papain (see Brocklehurst *et al.*, 1981a), ficin (Malthouse & Brocklehurst, 1976), actinidin (Brocklehurst *et al.*, 1981b) and papaya peptidase A (Baines & Brocklehurst, 1982). The close proximity (within 0.5 nm) of the thiol group and a histidine imidazole group has been demonstrated for papain and ficin by cross-linking experiments (see Lowe, 1976) and for papain and actinidin by X-ray crystallography (see below). No structural information about the active-centre region of papaya peptidase A, other than the presence of a cysteine

residue, is available, but the behaviour of this enzyme towards 2,2'-dipyridyl disulphide (Baines & Brocklehurst, 1982) is consistent with the presence of a thiol–imidazole interactive system. The only other ionizing group that has been found or suggested to be present in the immediate vicinity of these thiol–imidazole systems is the carboxy group of an aspartic acid residue (aspartic acid-158 in papain and aspartic acid-161 in actinidin). The crystal structures of only two of these enzymes, actinidin at 0.17 nm resolution (Baker, 1980) and papain at 0.28 nm resolution (Drenth *et al.*, 1971), have been determined. The catalytic sites appear to be very similar, in that they both appear to contain thiol–imidazole–asparagine amide interactive systems with the active-centre carboxy group too far away for direct interaction. For actinidin Baker (1980) reports that the carboxy group of aspartic acid-161 is turned away from the active centre, as in papain, so that it hydrogen-bonds with the main-chain amido group of alanine-139. In this position Baker (1980) reports that it is approx. 0.56 nm from the $\text{N}_{(\delta)}$ atom of histidine-162 and approx. 0.71 nm from the $\text{S}_{(\gamma)}$ atom of cysteine-25 and that rotation around $\text{C}_{(\alpha)}\text{--}\text{C}_{(\beta)}$ of aspartic acid-161 would not bring it any nearer.

Despite the indications from the crystal structures that the direct ionic interactions of these active centres might be restricted to a $-+$ diad (S^-/ImH^+) with a $-+-$ triad ($\text{S}^-/\text{ImH}^+/\text{CO}_2^-$) apparently excluded, a number of further points are worthy of note. (i) The reactivity of the papain thiol group in acidic media towards certain reactivity probes (Brocklehurst *et al.*, 1981a) is controlled, not merely by one pK_a value, approx. 4 [which could characterize the generation of a $-+$ system (S^-/ImH^+) from a $+$ system (SH/ImH^+)], but by two pK_a values each approx. 4. This additional kinetically significant pK_a of approx. 4 in papain could be associated with the ionization of the carboxy group of aspartic acid-158, and lack of evidence from analogous reactivity-probe experiments for the influence of similar additional groups in ficin (Malthouse & Brocklehurst, 1976) and actinidin (Brocklehurst *et al.*, 1981b) could be due to differences in the pK_a values of these carboxy groups. (ii) Some uncertainty about the exact structures of the catalytic sites of these enzymes remains because the actinidin structure was determined with crystals of oxidized enzyme and because the structure of papain was determined with crystals grown at high pH (see Brocklehurst *et al.*, 1981b, and references cited therein).

Attempts to detect the influence of carboxy groups on the reactivity of the catalytic-site thiol group by using two-protonic-state reactivity probes (Brocklehurst, 1982) have proved unsuccessful with all cysteine proteinases studied so far other than

papain. This could be perhaps because only papain happens to contain a carboxy group with a pK_a value approx. 4; the two-protonic-state-probe technique for the detection of such a perturbing group relies on a change in ionization state in a critical region at low pH. In the present paper a different type of probe technique has been employed, which should detect differences in solvation properties of the region around the catalytic site, including differences involving the postulated carboxy group even if its pK_a is very low. Thus, by studying the active centre in alkaline media, it can be ensured that both the thiol group and the postulated carboxy group will be essentially fully ionized and only the imidazolium ion will be changing its state of ionization (from positive to neutral). By using a pair of disulphide reactivity probes of similar size, one uncharged and the other negatively charged, it was hoped to ascertain whether the enzyme regions in the immediate vicinity of the catalytic sites are closely similar or whether substantial differences exist, e.g. whether papain is quite different from the other (perhaps closely similar) cysteine proteinases, a possibility suggested by probe work performed in acidic media. The probes used in the present work are Nbs_2^{2-} (I) and 4-Py-S-S-4-Py (II); 2-Py-S-S-2-Py is not suitable for this particular type of study because, unlike the 4,4'-isomer, it responds in an anomalous way to solvent effects (Malthouse & Brocklehurst, 1980).

Reactions of the thiol groups of the four cysteine proteinases with the two reactivity probes

All eight reactions studied under pseudo-first-order conditions with the probe reagent in excess

followed good first-order kinetics with respect to time to at least 80% reaction. All eight reactions were also first-order with respect to concentration in the probe reagent over the range of concentrations used. Typical plots that establish the order in probe reagent are shown in Fig. 1. The pH-dependence, mainly in alkaline media, of the second-order rate constants for all eight reactions are shown as logarithmic plots in Figs. 2 and 3. The characterizing parameters of these reactions (pK_a values and pH-independent rate constants, k) together with the ratios of the rate constants, $k_{Nbs_2^{2-}}/k_{4-Py-S-S-4-Py}$, are given Table 1.

The striking feature of the data in Table 1 is that, whereas the reactivities of all of the four enzyme thiolate anions towards the uncharged probe (4-Py-S-S-4-Py) are rather similar (with the papain thiolate anion being perhaps a little more reactive than the others), the reactivities towards the negatively charged probe (Nbs_2^{2-}) differ considerably. Actinidin is by far the most reactive towards

Fig. 1. Typical plots of first-order rate constant versus probe-reagent concentration for reactions performed under pseudo-first-order conditions with enzyme concentration $10\ \mu\text{M}$, at 25°C and at 10.1

(a) Reaction of ficin with Nbs_2^{2-} at pH 9.5. (b) Reaction of papaya peptidase A with 4-Py-S-S-4-Py at pH 9.7.

Fig. 2. *pH-dependence of the second-order rate constant (k) for the reactions of Nbs_2^{2-} with (a) actinidin, (b) ficin, (c) papain and (d) papaya peptidase A at 25°C and at I 0.1*

The concentrations of the reagent and the enzymes are given in the Materials and methods section. The pH-dependence of k in each case is of the form:

$$k = \frac{\bar{k}_{\text{XH}}}{\left(1 + \frac{K}{[\text{H}^+]}\right)} + \frac{\bar{k}_{\text{X}}}{\left(1 + \frac{[\text{H}^+]}{K}\right)}$$

The values of the characterizing parameters obtained as described in the Materials and methods section are: for (a) $\bar{k}_{\text{XH}} \leq 2.3 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\bar{k}_{\text{X}} = 3.58 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\text{p}K = 9.61$; for (b) $\bar{k}_{\text{XH}} = 2.00 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\bar{k}_{\text{X}} = 2.82 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\text{p}K = 8.61$; for (c) $\bar{k}_{\text{XH}} = 48 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\bar{k}_{\text{X}} = 4.11 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\text{p}K = 8.62$; for (d) $\bar{k}_{\text{XH}} = 5.20 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\bar{k}_{\text{X}} = 1.52 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\text{p}K = 8.67$. The data are displayed as logarithmic plots in which the line of zero slope at high pH ($k = \bar{k}_{\text{X}}$) intersects the line of slope + 1 at $\text{pH} = \text{p}K$; the line of zero slope at the lower pH plateau is $k = \bar{k}_{\text{XH}}$.

Table 1. *Reactivities of the catalytic-site thiolate anions of some cysteine proteinases and the thiolate anion of 2-mercaptoethanol towards an uncharged disulphide probe and towards a negatively charged disulphide probe at 25°C and at I 0.1*

The rate constants, $\bar{k}$, characterize the reactions of the thiolate anions when the active-centre carboxy groups are ionized and the catalytic-site imidazole groups are uncharged. The molecular $\text{p}K_{\text{a}}$ value is associated with the protonic dissociation of the interactive system to provide the $\text{S}^-/\text{Im}/\text{CO}_2^-$ state of the active centre.

Location of the thiolate anion	$10^{-3}\bar{k} \text{ (M}^{-1} \cdot \text{s}^{-1}\text{)}$		$\text{p}K_{\text{a}}$		$R = \frac{\bar{k}_{\text{Nbs}_2^{2-}}}{\bar{k}_{4\text{-Py-S-S-4-Py}}}$
	Nbs_2^{2-}	4-Py-S-S-4-Py	Nbs_2^{2-}	4-Py-S-S-4-Py	
Actinidin	358	12.2	9.6	9.6	29
Papaya peptidase A	15.2	19.1	8.7	8.2	0.80
Ficin	2.8	15.7	8.6	8.7	0.18
Papain	0.4	27.5	8.6	8.9	0.015
2-Mercaptoethanol*	120	139	9.5	9.8	0.86

* Malthouse & Brocklehurst (1980).

the anionic probe, and there are large differences between the reactivities of the other enzymes, the order of reactivity being actinidin > papaya peptidase A > ficin > papain; $\bar{k}_{\text{actinidin}}/\bar{k}_{\text{papaya peptidase A}} = 23.6$, $\bar{k}_{\text{papaya peptidase A}}/\bar{k}_{\text{ficin}} = 5.4$, $\bar{k}_{\text{ficin}}/\bar{k}_{\text{papain}} = 7$

and $\bar{k}_{\text{actinidin}}/\bar{k}_{\text{papain}} = 895$. With the exception of the reaction of actinidin with Nbs_2^{2-} , all of the other reactivities are much lower than those of the thiolate anion of the reference low- M_r thiol, 2-mercaptoethanol. Some of the lower reactivity is no doubt due

Fig. 3. pH-dependence of the second-order rate constants (k) for the reactions of 4-Py-S-S-4-Py with (a) actinidin, (b) ficin, (c) papain and (d) papaya peptidase A at 25°C and at 10.1

The pH-dependence is described by the equation given in Fig. 2 legend. For (a) $\bar{k}_{\text{XH}} = 1.18 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\bar{k}_{\text{X}} = 1.22 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\text{pK} = 9.61$; for (b) $\bar{k}_{\text{XH}} = 400 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\bar{k}_{\text{X}} = 1.57 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\text{pK} = 8.67$; for (c) $\bar{k}_{\text{XH}} = 630 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\bar{k}_{\text{X}} = 2.75 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\text{pK} = 8.70$; for (d) $\bar{k}_{\text{XH}} = 500 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\bar{k}_{\text{X}} = 1.91 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\text{pK} = 8.15$. For (a) it was not possible to determine the pK_{a} by using 4-Py-S-S-4-Py because of the loss of absorbance of pyridine-4-thione at high pH; the continuous lines are shown to intersect at $\text{pH} = 9.61$, which is the value of the pK_{H} of actinidin determined kinetically by using Nbs_2^{2-} as a reactivity probe (see Fig. 2a).

to steric effects, but the effects of at least gross steric differences in the transition states and/or in adsorptive complexes can be eliminated by consideration of the ratio $R = \bar{k}_{\text{Nbs}_2^{2-}} / \bar{k}_{4\text{-Py-S-S-4-Py}}$. The reactivities of the 2-mercaptoethanol anion towards the neutral and anionic probes are quite similar, with $R = 0.86$. Like the low- M_r thiol, papaya peptidase A does not discriminate markedly between the probes, with $R = 0.80$. By contrast, the other enzymes discriminate markedly, with R for actinidin being $\gg 1$, and the R values for the other enzymes being $\ll 1$; $R_{\text{actinidin}} / R_{\text{papaya peptidase A}} = 36$, $R_{\text{ficin}} /$

$R_{\text{papaya peptidase A}} = 0.22$, $R_{\text{papain}} / R_{\text{papaya peptidase A}} = 0.019$, and the biggest difference in R values, $R_{\text{actinidin}} / R_{\text{papain}}$, being 1933.

The gradation in properties of these active centres does not correlate with the pI values of the enzymes, actinidin having the lowest value ($\text{pI} 3.1$; McDowell, 1970) and papaya peptidase A the highest (pI approx. 11), with those for papain and ficin lying in between (pI of both approx. 9) (see, e.g., Brocklehurst *et al.*, 1981a). The factors responsible, therefore, are likely to be localized in the immediate active-centre environment and may be of con-

siderable importance in the solvation of ionic transition states.

The order of the R values is the same as the order of the reactivities towards Nbs_2^{2-} , emphasizing that the main effect probably derives from the influence of the negative charge of the probe on its interaction with the catalytic-site environment, although specific steric effects that derive from the different substituents on the two probes cannot definitely be ruled out. The marked differences in the R values of Table 1 suggest (a) that the catalytic-site solvation properties of these enzymes are very different from each other and (b) that this group of functionally related enzymes does provide an opportunity to study the effects of structural differences in active centres on reactivity characteristics of constituent nucleophiles and on catalytic competence. It is notable that the catalytic-site environments of actinidin and papain appear to be so different from each other in terms of solvation factors affecting the nucleophilic competence of the catalytic site in view of the close similarities in structure suggested by the X-ray-diffraction studies.

The fact that papain is found to be at the extreme of this series of four cysteine proteinases at which the repulsion of the anionic probe is greatest might indicate a particular disposition of the active-centre carboxy and thiol groups not found with any of the other cysteine proteinases, and this might explain the unique behaviour of papain towards other reactivity probes in acidic media (see Brocklehurst *et al.*, 1981a).

The wide variation in reactivity characteristics shown in Table 1 emphasizes that papain should not necessarily be regarded as archetypal of cysteine proteinases in general, and that at least certain aspects of the structure and mechanism of action of a given cysteine proteinase need to be investigated by using the particular enzyme rather than relying on the results of analogous studies on papain.

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