Evidence that the active centre of chymopapain A is different from the active centres of some other cysteine proteinases and that the Brønsted coefficient ($\beta_{\text{nuc.}}$) for the reactions of thiolate anions with 2,2'-dipyridyl disulphide may be decreased by reagent protonation

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The active centres of chymopapains A and B (jointly designated EC 3.4.22.6) and papaya (*Carica papaya* L.) peptidase A were investigated by using 2,2'-dipyridyl disulphide and 5,5'-dithiobis-(2-nitrobenzoic acid) as thiol-specific reactivity probes. Whereas the first active-centre pK_a values for chymopapain B and papaya peptidase A are less than 5, as is the case for papain (EC 3.4.22.2) and ficin (EC 3.4.22.3), that for chymopapain A is about 6.8. The reason why the reactions of thiols of pK_a approx. 6.5 with 2,2'-dipyridyl disulphide are essentially pH-independent in the pH range around the thiol pK_a is delineated. The value of the Brønsted coefficient ($\beta_{\text{nuc.}}$) for the reactions of thiolate ions with the 2,2'-dipyridyl disulphide monocation appears to be smaller than its value for the corresponding reactions with the neutral disulphide.

The dried latex of the fruit of the papaya plant (*Carica papaya* L.) contains at least four cysteine proteinases, namely papain (EC 3.4.22.2) chymopapains A and B (designated jointly as EC 3.4.22.6) and papaya peptidase A (see Ebata & Yasunobu, 1962; Cayle *et al.*, 1964; Kunimitsu & Yasunobu, 1967; Schack, 1967; Robinson, 1975). Extensive detailed investigation of the mechanisms of action of cysteine proteinases has been confined hitherto to papain, although limited study of cysteine proteinases from other biological sources has indicated a number of common mechanistic features, notably the transient existence of an acyl-enzyme intermediate involving the active-centre thiol group and the participation of a neighbouring histidine side chain in its formation and subsequent hydrolysis (see Lowe, 1976). More detailed study of other cysteine proteinases may help to ascertain the extent to which this group of enzymes operates by a common reaction mechanism and to identify possible variations. Study of a group of cysteine proteinases may help to delineate essential or optimal features of enzyme-catalysed hydrolysis of amide bonds and may be of interest in connection with the evolution of enzyme structure and function.

The present paper reports that whereas the

active-centre cysteine-histidine systems of papain, papaya peptidase A and chymopapain B may all be rather similar in that the first molecular pK_a values (pK_1) characteristic of the formation of the thiol-imidazole/thiolate-imidazolium interactive systems are all less than 5 (probably about 4), the active centre of chymopapain A may be substantially different from the others with $pK_1 \approx 6.8$.

Materials and methods

Many of the materials and general methods have been described previously, notably spectral characteristics of disulphide reactivity probes and their thiolysis products, buffers and determination of second-order rate constants (k) by stopped-flow spectral analysis and of the characterizing parameters of pH- k profiles by an optimization procedure (Brocklehurst & Little, 1973; Shipton & Brocklehurst, 1978).

Enzymes

Papain, chymopapains A and B and papaya peptidase A were prepared from spray-dried papaya latex (Powell & Scholefield, Liverpool L7 3JG, U.K.) by chromatography on SP-Sephadex as described by Baines & Brocklehurst (1979) (see also Robinson, 1975).

Abbreviations used: SP-, sulphopropyl; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid).

Stopped-flow kinetics

Kinetic runs were performed in aqueous buffers at 25°C, I 0.1, in a Durrum D110 stopped-flow spectrophotometer linked to a Tektronix 510 3N storage oscilloscope. One syringe contained an aqueous solution of activated, activator-free enzyme and the other a solution of 2,2'-dipyridyl disulphide or Nbs₂ in double-strength buffer. The initial concentrations of the reactants in the mixing chamber of the stopped-flow apparatus were: enzyme, 10–20 μM; disulphide reagent 100–250 μM. Reactions of 2,2'-dipyridyl disulphide were monitored at 343 nm and those of Nbs₂ at 412 nm.

In cases where the recorded progress curves were biphasic (reactions at pH values greater than 3 of the two thiol groups present in each of the chymopapains) the rate constants of the two phases were determined by using the method of Frost & Pearson (1961).

Results and discussion

One of the important characteristics of both papain and ficin is the existence in weakly acidic media of interactive thio-imidazole/thiolate-imidazolium systems ($\text{SH} \rightleftharpoons \text{Im} \rightleftharpoons \text{S}^- \rightleftharpoons \text{ImH}^+$), whose formation from (SH ImH^+) and deprotonation to ($\text{S}^- \text{Im}$) are characterized by apparent pK_a values of about 4 and 9 respectively (Malthouse & Brocklehurst, 1976; Shipton & Brocklehurst, 1978). The requirement in the acylation step of the catalytic act for both a nucleophilic S atom, and an imidazolium ion to act as a general acid catalyst, is in accord with the bell-shaped dependence on pH of $k_{\text{cat.}}/K_m$ (pK_a values approx. 4 and 9) (see Lowe, 1976).

The existence of such interactive active-centre systems can be demonstrated particularly clearly by using thiol-specific two-protonic-state electrophiles, such as 2,2'-dipyridyl disulphide, as reactivity probes. The existence of a nucleophilic state in acidic media ($\text{SH} \rightleftharpoons \text{Im} \rightleftharpoons \text{S}^- \rightleftharpoons \text{ImH}^+$) additional to the uncomplicated thiolate ion ($\text{S}^- \text{Im}$) that predominates at high pH is revealed by the shape of the profile of apparent second-order rate constant (k) against pH for reaction with 2,2'-dipyridyl disulphide (see Brocklehurst *et al.*, 1979a). The general profile shape consists of a prominent bell-shaped component in acidic media with a rate maximum at about pH 3–4, a rate minimum at about pH 6–7 and a higher-rate plateau at pH values above 9–10. This type of pH– k profile has been shown to be characteristic not only of the active centres of papain and ficin, but also of the active-centre model, benzimidazol-2-ylmethanethiol, which also produces molecular states containing nucleophilic S by two

stages of ionization (in this case with pK_a values of 5.6 and 8.3) (Brocklehurst *et al.*, 1979b).

The data in Table 1 show that, as in the reactions of 2,2'-dipyridyl disulphide with papain and ficin (Malthouse & Brocklehurst, 1976; Shipton & Brocklehurst, 1978), values of k for the reactions of this disulphide with the active-centre thiol groups of papaya peptidase A and chymopapain B increase with decrease in pH in the region pH 6–4. For all these four enzymes, pK_1 of the active-centre system is less than 5. Like papain, papaya peptidase A contains only one free thiol group per molecule, which is part of its active centre, whereas both chymopapain A and chymopapain B each contain two thiol groups per molecule, only one of which (a) is essential for catalytic activity and (b) reacts rapidly with 2,2'-dipyridyl disulphide at low pH (Baines & Brocklehurst, 1978).

In marked contrast with the form of pH-dependence of k for the reactions of 2,2'-dipyridyl disulphide with papain, ficin, papaya peptidase A and chymopapain B, Table 1 shows that k for the reaction of this disulphide with the active-centre thiol group of chymopapain A does not increase with decreasing pH in acidic media and is essentially constant in the pH range 3.5–5.5. At pH values below 3.5, k decreases according to $pK_a = 2.46$ (the pK_{11} of 2,2'-dipyridyl disulphide is 2.45; Brocklehurst & Little, 1973). At pH values about 5.5, k increases with increasing pH, but accurate values of k became more difficult to obtain by the method of Frost & Pearson (1961) because the rate of reaction of the inessential thio group becomes comparable with that of the essential thiol group.

The relatively rapid reaction at high pH presumably involves reaction of neutral 2,2'-dipyridyl disulphide with the uncomplicated thiolate ion of the deprotonated active centre. The nature of the reaction in the plateau region pH 3.5–5.5 is particularly noteworthy because it has no analogue in the pH– k profiles for the reactions of 2,2'-dipyridyl disulphide with the other four cysteine proteinases. A possible interpretation was suggested by the pH– k profile for the reaction of 2,2'-dipyridyl disulphide with thiophenol, which has a pK_a of approx. 6.5 (T. Stuchbury & K. Brocklehurst, unpublished work). For this reaction, k remains essentially constant over the pH range 3–8, i.e. a pH range that spans the thiol pK_a . Changes in the rates of reaction of 2,2'-dipyridyl disulphide with thiols in acidic media are determined by both the loss of nucleophilic thiolate anion consequent on its protonation and the increase in electrophilicity of the disulphide consequent on protonation of a pyridyl-ring N atom (Brocklehurst *et al.*, 1979b). If a particular thiol has a pK_a value such that the increase in the electrophilicity of 2,2'-dipyridyl disulphide (pK_a 2.45) consequent on protonation compensates more or less exactly for the

Table 1. *Second-order rate constants (k) for the reactions of the active-centre thiol groups of some cysteine proteinases with disulphide reactivity probes at 25°C and at I 0.1*

Probe reagent	Enzyme	pH	$10^{-3}k$ ($M^{-1}\cdot s^{-1}$)
2,2'-Dipyridyl disulphide	Chymopapain B	3.80	10.2
		4.15	9.0
		4.73	6.8
		5.26	3.2
		6.01	2.4
		6.50	1.5
	Papaya peptidase A	3.48	12.1
		4.03	7.2
		4.60	3.5
		5.55	0.6
		6.50	0.6
	Chymopapain A	1.23	0.2
		1.94	0.5
		2.51	1.2
		2.93	1.9
		3.18	2.3
		3.31	2.5
		3.71	2.4
		4.16	2.4
		4.88	2.3
		5.27	2.5*
5,5'-Dithiobis -(2-nitrobenzoic acid)	Chymopapain A	6.55	8.6
		7.15	15.4
		10.01	18.2
		5.45	4.5
		5.80	10.7
		6.49	20.2
		6.89	30.8
		7.65	45.0
		7.81	55.2
		8.98	54.1
		9.27	57.5†

* At pH values ≤ 5.27 , the pH-dependence of k for this reaction is given by $k = \bar{k}/(1 + [H^+]/K_a)$, where the pH-independent rate constant $\bar{k} = 2.46 \times 10^3 M^{-1}\cdot s^{-1}$ and $pK_a = 2.46$.

† The pH-dependence of k for this reaction at pH values ≤ 9.27 is given by $k = \bar{k}/(1 + [H^+]/K_a)$, where $\bar{k} = 5.75 \times 10^4 M^{-1}\cdot s^{-1}$ and $pK_a = 6.77$.

loss of nucleophilic thiolate ion, k will remain essentially constant over the pH range around the thiol pK_a . The results obtained with thiophenol suggested that the thiol pK_a that affords such compensation might be about 6.5. Since the pK_a of the 2,2'-dipyridyl disulphide monocation (2PDSH⁺) is about 2.5, the rate enhancement factor, F ($F = \tilde{k}_+/\tilde{k}_0$, where $\tilde{k}_+$ is the rate constant for the reaction of thiolate ion with the cationic reagent and $\tilde{k}_0$ is the rate constant for the reaction with the neutral disulphide reagent) would have to be about 1×10^4 i.e. $10^{(pK_{RSH} - pK_{2PDSH^+})}$ to account for the pH-independence of k for the thiophenol reaction. The value of F for the reaction of 2,2'-dipyridyl disulphide with 2-mercaptoethanol (pK_a 9.6) is 0.2×10^4 (Brocklehurst *et al.*, 1979b). It seems possible, however, that F might be larger for

reactions of thiols with low pK_a values than for thiols with high pK_a values. This would be so if the value of the Brønsted coefficient, β_{nuc} , (see Hupe & Jencks, 1977) were lower for the reactions of thiolate anions with the 2,2'-dipyridyl disulphide monocation than the value ($\beta_{nuc} = 0.24$; see Brocklehurst *et al.*, 1979b) for the reactions of thiolate anions with the neutral disulphide. The postulate of a lower value of β_{nuc} for the reactions of the highly reactive cationic disulphide, implying a transition state relatively early on the reaction co-ordinate, where the charge on the attacking nucleophile has been little changed (see Fersht & Jencks, 1970), does not seem unreasonable. The lower limit of F (6.4×10^3) for benzimidazol-2-ylmethanethiol (pK_1 5.6) may be calculated by using the value of K ($2.5 \times 10^3 M^{-1}\cdot s^{-1}$) in the plateau region at pH 6–7 of Fig. 1(b) of

Brocklehurst *et al.* (1979b). This result is consistent with the prediction from the data for the thiophenol reaction that F for a thiol of pK_a approx. 6.5 should be of the order of 10^4 .

To test the postulate that pK_I of the active centre of chymopapain A may be about 6.5 and is not revealed by the pH- k profile for the reaction of 2,2'-dipyridyl disulphide because of the compensation provided by the formation of the more highly electrophilic cationic form of the disulphide at the pH is decreased, we studied the reaction of the active-centre thiol group of chymopapain A with Nbs_2 , a disulphide reagent that does not possess the ability to increase its electrophilicity by protonation. The change in k predicted for this reaction in the pH range 5–9 is shown by the data in Table 1, and is dependent on a pK_a of 6.8. It is noteworthy in this connection that Kanazawa *et al.* (1976) reported that a single histidine residue (pK_a approx. 7) is essential for the activity of chymopapain A.

An active centre characterized by pK_I approx. 7 is usually considered to be typical of the serine proteinases rather than of the cysteine proteinases. One consequence of this relatively high pK_a for chymopapain A is that optimal catalytic activity may be exhibited over a more restricted range of pH, around neutrality, than that which obtains for other cysteine proteinases.

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