Evidence for a two-state transition in papain that may have no close analogue in ficin

Differences in the disposition of cationic sites and hydrophobic binding areas in the active centres of papain and ficin

Keith BROCKLEHURST and J. Paul G. MALTHOUSE

Department of Biochemistry and Chemistry, Medical College of St. Bartholomew's Hospital, University of London, Charterhouse Square, London EC1M 6BQ, U.K.

(Received 18 February 1980/Accepted 6 June 1980)

The kinetics of the reactions of the active-centre thiol groups of papain (EC 3.4.22.2) and ficin (EC 3.4.22.3) with the two-protonic-state reactivity probes 2,2'-dipyridyl disulphide, n-propyl 2-pyridyl disulphide and 4-(*N*-aminoethyl 2'-pyridyl disulphide)-7-nitrobenzo-2-oxa-1,3-diazole (compound I) were studied over a wide range of pH. Differences between the reactivities of ficin and papain towards the cationic forms of the alkyl 2-pyridyl disulphide probes suggest that ficin contains a cationic site without exact analogue in papain, and the striking difference in the shapes of the pH-rate profiles for the reactions of the two enzymes with compound (I) suggests differences in the mobilities or dispositions of the active-centre histidine imidazole groups with respect to relevant hydrophobic binding areas. The evidence from reactivity-probe studies that the papain catalytic mechanism involves substantial repositioning of the active-centre imidazole group during the catalytic act does not apply also to ficin. If ficin contains an aspartic acid residue analogous to aspartic acid-158 in papain, the pK_a of its carboxy group is probably significantly lower than the pK_a of the analogous group in papain.

Although extensive detailed investigation of the mechanism of action of the cysteine proteinases has been confined hitherto to papain (EC 3.4.22.2), limited study of some other cysteine proteinases 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. This type of study should help to delineate essential or optimal features in enzyme-catalysed hydrolysis of amide bonds, and may be of interest in connection with the evolution of enzyme structure and function.

A central problem concerns the way in which the essential proton transfers are effected and coupled to other bond-making and bond-breaking steps to permit ready acylation by substrate of the active-centre thiol group. The position and mobility of the active-centre histidine imidazole group and the existence and conformation in other enzymes of

residues analogous to aspartic acid-158 in papain are prime targets for experimental study in this connection. Indications exist that there may be differences in the relative positions in space of active-centre residues in some cysteine proteinases. 1,3-Dibromoacetone, which cross-links the active-centre thiol and imidazole group in papain, ficin (EC 3.4.22.3) and bromelain (EC 3.4.22.4) (see Lowe, 1976), reacts only with the active-centre thiol group of streptococcal proteinase and fails to cross-link with the putative active-centre imidazole group (Kortt & Liu, 1973). Also, a difference in the relative positions of the thiol and imidazole groups in papain and in actinidin (the cysteine proteinase from *Actinidia chinensis*), which was revealed by the 0.28 nm-resolution electron-density map of actinidin (Baker, 1977), may account for a difference in the pH-dependences of the rates of reaction of the two enzymes with a cationic reactivity probe (Brocklehurst *et al.*, 1979*b*). There is particular interest currently in the possibility that substantial repositioning of the side chain of histidine-159 of papain may occur during the catalytic act to meet postulated stereoelectronic preferences (Brocklehurst & Malthouse, 1978; Brocklehurst *et al.*, 1979*a,c*;

Angelides & Fink, 1978, 1979a,b). This type of structural change, possibly involving rotation around both the $C_{(\alpha)}-C_{(\beta)}$ and $C_{(\beta)}-C_{(\gamma)}$ bonds of the histidine side chain, appears to have no analogy in serine proteinase mechanisms (see, e.g., Blow, 1976; Matthews *et al.*, 1977; Petkov *et al.*, 1978; but see James, 1980).

In the present paper, differences in the behaviour of the active centres of papain and ficin towards reactivity probes designed to characterize relative dispositions of nucleophilic and proton distributing sites are reported and related to possible variations in catalytic mechanism.

Materials and methods

Ficin (EC 3.4.22.3) was prepared from the dried latex of *Ficus glabrata* as described by Malthouse & Brocklehurst (1976). The synthesis of n-propyl 2-pyridyl disulphide was described by Shipton & Brocklehurst (1978). The synthesis of 4-(*N*-aminoethyl 2'-pyridyl disulphide)-7-nitrobenzo-2-oxa-1,3-diazole (compound I) was described by Stuchbury *et al.* (1975).

Other materials and methods were as described previously, notably spectral characteristics of pyridyl disulphides 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; Stuchbury *et al.*, 1975; Shipton & Brocklehurst, 1978). Kinetic runs were performed in aqueous buffers and in some cases in 13.4% (v/v) acetonitrile at 25°C at 10.1 in a Durrum D110 stopped-flow spectrophotometer linked to a Tektronix 510 3N storage oscilloscope. All solutions were degassed as a routine before stopped-flow experiments were performed. One syringe contained an aqueous solution of activated activator-free ficin, and the other a solution of compound (I), n-propyl 2-pyridyl disulphide or 2,2'-dipyridyl disulphide in double-strength buffer, which in some runs contained 26.8% (v/v) acetonitrile. The initial concentrations of the reactants in the mixing chamber of the stopped-flow apparatus were ficin, 10–14 μM , and disulphide reagent, 10–140 μM . Reactions were monitored at 343 nm.

Results and discussion

Two-state model for papain and possibilities for differences in mechanism between papain and ficin

The suggestion that catalytic advantage could result from the movement of the side chain of histidine-159 in papain (Brocklehurst & Malthouse, 1978; Angelides & Fink, 1978) has received support from kinetic analysis of the reactions of papain with ester and anilide substrates at sub-zero

temperatures (Angelides & Fink, 1978, 1979a,b), and with two-protonic-state reactivity probes, notably compound (I) (Brocklehurst *et al.*, 1979a,c). In terms of the detailed mechanism discussed by Angelides & Fink (1979a), papain exists at pH values around 6 mainly as the DOWN conformer E' (Scheme 1), in which the imidazole side chain of histidine-159 no longer occupies the location deduced from the X-ray-crystallographic data of Drenth *et al.* (1976) as it does in the UP conformer E (Scheme 1), considered to be a minor component at pH 6. Isomerization of E to E' is considered to involve rotation of the imidazole group of histidine-159 about its α - β axis by 79° and about its β - γ plane by 35° and repositioning of the carboxy side chain of aspartic acid-158 by rotation of $C_{(\alpha)}-C_{(\beta)}$ by 3° and of $C_{(\beta)}-C_{(\gamma)}$ by 68°. Protonation of either of the anionic side chains shown in E' or deprotonation of the imidazolium ion should promote isomerization from enzyme species with DOWN conformations to enzyme species with UP conformations (see Scheme 1), and the UP conformation should predominate at pH values below 4 and above 9.

The UP-DOWN transition of Scheme 1, which is the central feature of the mechanism, requires the presence of a carboxy group in the active centre as well as the thiol and imidazole groups. The question of whether a carboxy group equivalent to that of aspartic acid-158 in papain exists in the ficin active centre remains unresolved. Husain & Lowe (1970b) isolated the peptide containing the active-centre histidine residue from ficin, and reported the adjacent residue to be an aspartic acid residue, as in papain. On the other hand, studies on reactions of ficin with reactivity probes (Malthouse & Brocklehurst, 1976; Shipton *et al.*, 1976) have been interpreted to suggest that ficin may lack a carboxy group conformationally equivalent to that of aspartic acid-158 of papain. Further interpretation of reactivity-probe data presented previously, together with interpretation of new data on reactions of other reactivity probes (Tables 1–3), are shown in the present paper to support the presence of an UP-DOWN transition in papain and to suggest that the active centre of ficin may be more ordered than that of papain and less mobile, at least in acidic media.

Differences in the forms of pH-dependence of the rate of reaction of the two-protonic-state reactivity probe 2,2'-dipyridyl disulphide with the active-centre thiol groups of papain and ficin respectively: possibility of a cationic side-chain in the ficin active centre with no exact analogue in papain

Before presentation and discussion of the new data obtained for the reactions of ficin with two other two-protonic-state reactivity probes, it is useful

Scheme 1. Schematic representation of the active centre of papain, showing the four functional groups and the possibilities for interaction between them, including those that could result from movement of the imidazole group between the UP and DOWN conformations

The numbers refer to the following active-centre residues: cysteine-25, aspartic acid-158, histidine-159 and asparagine-175. For simplicity only a selection of the stepwise deprotonations of the fully protonated active centre is shown. DOWN conformations are shown only for the form in which the imidazolium ion could be bound by the two anionic sites and for the form generated by its deprotonation, which would presumably have a finite existence before isomerization to the UP conformation. It seems possible that forms generated by protonation of at least one of the anionic sites in $S^- \cdots ImH^+ \cdots CO_2^-$ (DOWN) might also exist. Possible hydrogen-bonding or electrostatic interactions are denoted by $\cdots$. Although Angelides & Fink (1979*a,b*) appear to assume that interaction between the side chains of cysteine-25 and histidine-159 does not occur in UP conformations, reactivity-probe studies suggest that it may do so (Shipton *et al.*, 1976; and see the text). Isomerization of the 'UP' conformer (E) to the 'DOWN' conformer (E') is considered to involve rotation of the imidazole side chain of histidine-159 about its α - β axis by 79° and about its β - γ plane by 35° (see Angelides & Fink, 1979*a*).

to consider further, in the light of the two-state model for the papain active centre (see above), data presented before evidence for the two-state model had been obtained.

Although the profiles of apparent second-order rate constant (k) against pH for the reactions of 2,2'-dipyridyl disulphide with papain (Shipton & Brocklehurst, 1978) and with ficin (Malthouse & Brocklehurst, 1976) are of similar general shape, the profiles are very different in important respects that suggest significant differences in active-centre structure between the two enzymes. The general profile

shape consists of a striking bell-shaped component in acidic media with a rate maximum at about pH 3–4, a rate minimum in a shallow plateau at about pH 6–7 and a somewhat higher rate plateau at pH values above 9–10. The characterizing parameters for these profiles (apparent pK_a values and pH-independent second-order rate constants, $\bar{k}$) are presented in Table 1 together with analogous parameters for reactions of the enzymes with some other reactivity probes. Because the bell-shaped component of the pH- k profile for the papain reaction in acidic media does not reflect the pK_a

Table 1. *Characteristics of the reactions of the active-centre thiol groups of papain and ficin with three reactivity probes each in three protonic states (X, XH and XH₂) at 25°C at 10.1*

The pH-independent rate constants ($\bar{k}_x - \bar{k}_{xH}$) relate to eqn. (1). For the XH₂ states of the reactions of ficin with 2,2'-dipyridyl disulphide and with n-propyl 2-pyridyl disulphide the values of the rate constant for the reaction of the enzyme with the monocationic form of the reagent [$\bar{k}_{xH_2}$ of eqn. (2)] are given in parentheses. Parameters characteristic of the reactions of ficin with (a) n-propyl 2-pyridyl disulphide, (b) compound (I) and (c) 2,2'-dipyridyl disulphide in 13.4% acetonitrile were determined in the present work; those characteristic of the other reactions are taken from: Shipton & Brocklehurst (1978) for papain/2,2'-dipyridyl disulphide and papain/n-propyl 2-pyridyl disulphide; Brocklehurst *et al.* (1979a) for papain/compound (I) and papain/2,2'-dipyridyl disulphide in 13.4% acetonitrile; Malthouse & Brocklehurst (1976) for ficin/2,2'-dipyridyl disulphide in water. Abbreviation: N.D., not determined.

Enzyme	Reactivity probe	Solvent	pK _I	pK _{II}	pK _{III}	$\bar{k}_{xH_2}$ (M ⁻¹ . s ⁻¹)	$\bar{k}_{xH}$ (M ⁻¹ . s ⁻¹)	$\bar{k}_x$ (M ⁻¹ . s ⁻¹)	$\bar{k}_x/\bar{k}_{xH}$	$\bar{k}_{xH_2}/\bar{k}_{xH}$
Papain	2,2-Dipyridyl disulphide	Water	3.85	3.90	8.8	42000	850	1700	2.0	49
Ficin	2,2'-Dipyridyl disulphide	Water	2.42	3.82	8.6	111000 (2.9 × 10 ⁶)	400	2230	5.6	278
Papain	n-Propyl 2-pyridyl disulphide	Water	3.86	3.89	8.8	131000	2400	5000	2.1	55
Ficin	n-Propyl 2-pyridyl disulphide	Water	3.0	3.8	8.6	24000 (6 × 10 ⁵)	1100	2900	2.6	22
Papain	Compound (I)	13.4% acetonitrile	3.5	8.7-8.95	—	0	51000	<20000	—	—
Papain	2,2'-Dipyridyl disulphide	13.4% acetonitrile	3.47	3.73	N.D.	11856	430	N.D.	—	28
Ficin	Compound (I)	13.4% acetonitrile	N.D.	N.D.	N.D.	~30000	14000	~30000	2	2
Ficin	2,2'-Dipyridyl disulphide	13.4% acetonitrile	N.D.	N.D.	N.D.	~10000	400	~1500	4	25

Scheme 2. Kinetic models for the reactions of an enzyme, $E\text{--}EH_3$, with a two-protonic-state reactivity probe, $R + RH^+$. K_e , K_{e1} – K_{e111} and K_r are molecular acid dissociation constants and $\tilde{k}_x$ – $\tilde{k}_{\text{XH}_3}$, etc. are pH-independent rate constants. In using Scheme 1(c) to describe reactions of papain and ficin with 2,2'-dipyridyl disulphide, the reactive components of the states characterized by $\tilde{k}_{\text{XH}_1}$ and $\tilde{k}_{\text{XH}_2}$ are considered to contain an undissociated carboxy group and those of the states characterized by $\tilde{k}_{\text{XH}_2}^*$ and $\tilde{k}_{\text{XH}}$ a carboxylate anion. Free protons and formal charges of the various ionization states, except for RH^+ , are omitted for clarity.

value of the 2,2'-dipyridyl disulphide monocation (pK_a 2.45; Brocklehurst & Little, 1973) but rather reflects two positively co-operative pK_a values close to 4, we described the pH-dependence of k in terms of Scheme 2(a) by eqn. (1) (Brocklehurst & Little, 1972; Brocklehurst, 1974; Shipton & Brocklehurst, 1978), which contains molecular acid dissociation constants associated with only the enzyme molecule:

$$k = \frac{\tilde{k}_{\text{XH}_2}}{1 + \frac{[\text{H}^+]}{K_{e1}} + \frac{K_{e11}}{[\text{H}^+]}} + \frac{\tilde{k}_{\text{XH}}}{1 + \frac{[\text{H}^+]^2}{K_{e1} \cdot K_{e11}} + \frac{[\text{H}^+]}{K_{e11}} + \frac{K_{e111}}{[\text{H}^+]}} + \frac{\tilde{k}_{\text{X}}}{1 + \frac{[\text{H}^+]}{K_{e111}}} \quad (1)$$

It is probable that study of pH-dependence of rates of covalent modification reactions that obey second-order kinetics provides pK_a values of the reactant state uncomplicated by either enzyme–reagent association or by extraneous rate constants (Brocklehurst & Dixon, 1977; Brocklehurst, 1979). The increase in k in acidic media was attributed to assistance to the reaction of neutral 2,2'-dipyridyl disulphide provided by the un-ionized carboxy group of aspartic acid-158, as envisaged also for the reaction of papain with 4-chloro-7-nitrobenzofurazan (Allen & Lowe, 1973; Shipton *et al.*, 1976).

By contrast with the papain reaction, the reaction of ficin with 2,2'-dipyridyl disulphide does appear to reflect the pK_a of the 2,2'-dipyridyl disulphide monocation, and the bell-shaped component of the pH– k profile was described in terms of Scheme 2(b) by eqn. (2) (Malthouse & Brocklehurst, 1976):

$$k = \frac{\tilde{k}_{\text{XH}_2} \cdot \left(\frac{K_e}{K_e + K_r} \right)}{\frac{[\text{H}^+]}{K_e + K_r} + \frac{K_e \cdot K_r / (K_e + K_r)}{[\text{H}^+]}} \quad (2)$$

The value of the numerator of eqn. (2) corresponds to the experimentally determined value of the pH-independent rate constant for the reaction associated with the bell-shaped component at low pH. This value needs to be multiplied by the assembly of acid dissociation constants $[(K_e + K_r)/K_e]$ to provide the value of the rate constant, $\tilde{k}_{\text{XH}_2}^*$, characteristic of the reaction of the interactive thiol–imidazole pair of ficin

with the 2,2'-dipyridyl disulphide monocation.

The pH-dependence of the reaction of ficin with 2,2'-dipyridyl disulphide follows closely the theoretical predictions for a reaction of a thiol compound in which the thiol group undergoes ionization in two stages (characterized by pK_a values 3.9 and 8.6) with a two-protonic-state electrophile (pK_a 2.45) (for a description of the theoretical predictions of profile shape and characteristics see Brocklehurst *et al.*,

1979a). By contrast, the corresponding reaction of papain does not obey this simple model in the pH range below 6 [see Malthouse & Brocklehurst (1976) and Fig. 1 for a comparison of the two pH- k profiles]. The deviation exhibited by the papain reaction is manifested by much lower reactivity in the bell-shaped component in acidic media (see Fig. 1 and Table 1) and by the existence of a narrower bell in which the two pK_a values are closely similar (approx. 3.9) and characteristic of positively co-operative protonic dissociations. In view of the high reactivity of the ficin active centre towards the 2,2'-dipyridyl disulphide monocation, it no longer seems appropriate to ascribe reaction in the bell-shaped component of the papain reaction to general acid catalysis of the reaction of the neutral probe molecule. The faster reaction predicted for the cationic form of the probe molecule by analogy with the ficin reaction would be expected to mask the contribution by the general acid-catalysed reaction involving the carboxy group of aspartic acid-158. It seems more likely that the deviation of the papain reaction from the behaviour expected for a simple two-nucleophilic/two-electrophilic-state reaction (Brocklehurst *et al.*, 1979a) might be due to inhibition of the reaction of papain with the 2,2'-dipyridyl disulphide monocation occasioned by protonation of the carboxylate ion of aspartic acid-158. In terms of the version of the two-state conformational model of the papain active centre described by Angelides & Fink (1979a), protonation of this carboxylate ion would result in the return of the imidazolium ion of histidine-159 from the DOWN conformation, in which it interacts electrostatically with the carboxylate ion of aspartic acid-158, to the UP conformation (see Scheme 1), with consequent loss of or decrease in nucleophilic character in the sulphur atom of cysteine-25. The increase in reactivity of the papain sulphur atom towards 4-chloro-7-nitrobenzofurazan in acid media (Shipton *et al.*, 1976) compels the view that at least some nucleophilic character is retained by the sulphur atom when the aspartic acid-158 carboxylate ion is protonated, but a substantial decrease in nucleophilic character in the UP conformation cannot be excluded.

To investigate the possibility that the difference in the forms of pH-dependence observed for the reaction of 2,2'-dipyridyl disulphide with papain and ficin might be explained by inhibition of the papain reaction by protonation of aspartic acid-158, we evaluated both reactions in terms of the more general kinetic model of Scheme 2(c), where account is taken of reaction of both neutral (R) and cationic (RH⁺) forms of the 2,2'-dipyridyl disulphide with enzyme forms in which a neighbouring carboxy group is (a) ionized and (b) un-ionized.

In terms of Scheme 2(c), the pH-dependence of k

(when $K_r \gg K_{ei}$; $pK_r = 2.45$ and $pK_{ei} = 3.8$ – 3.9) for the reaction in acidic media is given by eqn. (3):

$$k = \frac{\tilde{k}_{XH_3} \cdot \left(\frac{K_{ei}}{K_r} \right)}{1 + \frac{[H^+]}{K_r} + \frac{K_{ei}}{[H^+]} + \frac{K_{ei} \cdot K_{eII}}{[H^+]^2}} + \frac{\tilde{k}_{XH_2} + k_{XH_2}^* \cdot \left(\frac{K_{eII}}{K_r} \right)}{1 + \frac{[H^+]}{K_{ei}} + \frac{K_{eII}}{[H^+]} + \frac{[H^+]^2}{K_{ei} \cdot K_r}} + \frac{\tilde{k}_{XH}}{1 + \frac{[H^+]}{K_{eII}} + \frac{[H^+]^2}{K_{ei} \cdot K_{eII}} + \frac{[H^+]^3}{K_{ei} \cdot K_{eII} \cdot K_r}} \quad (3)$$

Profiles of k against pH for the reactions of 2,2'-dipyridyl disulphide with both papain and ficin were plotted, with $pK_r = 2.45$, $pK_{ei} = pK_{eII} = 3.85$ and $\tilde{k}_{XH} = 850 \text{ M}^{-1} \cdot \text{s}^{-1}$ being used for the papain reaction and $\tilde{k}_{XH} = 400 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the ficin reaction (see Table 1) and various values of the other parameters of eqn. (3). For the ficin reaction, a good fit to the experimental data is obtained by using $\tilde{k}_{XH_2} = 400 \text{ M}^{-1} \cdot \text{s}^{-1}$ (or $\tilde{k}_{XH_2} = 0$) and $\tilde{k}_{XH_3} = \tilde{k}_{XH_2}^* = 2.27 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ (see Fig. 1a). For these values, the first term of eqn. (3) is dominant at pH values less than 3.8, and conventional analysis of the plotted bell provides $pK_I \approx 2.4$, $pK_{II} \approx 3.8$ and $\tilde{k} \approx 1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. For the papain reaction, a good fit to the experimental data (i.e. for the bell, $pK_I \approx pK_{II} \approx 3.85$ and $\tilde{k} \approx 4 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$; see Table 1) is obtained by using $k_{XH_2}^* \approx 1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ (i.e. only about a factor of 2 lower than the value for the ficin reaction), provided that the second term of eqn. (3) is allowed to dominate the profile (e.g. $\tilde{k}_{XH_3} = 1 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\tilde{k}_{XH_2} = 1 \text{ M}^{-1} \cdot \text{s}^{-1}$) (see Fig. 1b). Thus the data can accommodate the postulate that the reactivities of papain and ficin towards the 2,2'-dipyridyl disulphide monocation are, after all, rather similar, provided that it is assumed that protonation of the carboxylate ion of aspartic acid-158 of papain greatly inhibits the reaction, whereas no analogous inhibitory process occurs in the ficin reaction. This lack of inhibition by a carboxy group in the reaction of ficin with 2,2'-dipyridyl disulphide correlates with the lack of general acid catalysis in the reaction of ficin with 4-chloro-7-nitrobenzofurazan, which contrasts markedly with the acid catalysis found in the corresponding reaction of papain (Shipton *et al.*, 1976). There seem to be three possible types of explanation for the different reactivity characteristics displayed by the active centres of papain and ficin in weakly acidic media.

(1) The residue analogous in the ficin sequence to

Fig. 1. Demonstration that the reactions of 2,2'-dipyridyl disulphide with both papain and ficin in acidic media can be described in terms of the model of Scheme 2(c) involving reaction of both enzymes with the 2,2'-dipyridyl disulphide monocation and marked inhibition of the papain reaction by protonation of the carboxylate anion of aspartic acid-158

(a) Reaction of ficin with 2,2'-dipyridyl disulphide: the points (●) are the experimental values reported by Malthouse & Brockelhurst (1976) and the continuous line is theoretical for eqn. (3) of the text in which $pK_r = 2.45$, $pK_{el} = pK_{eII} = 3.85$, $k'_{XH} = k'_{XH_2} = 400 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k'_{XH_3} = k'_{XH_2} = 2.27 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. (b) Reaction of papain with 2,2'-dipyridyl disulphide: the points (■) are the experimental values reported by Shipton & Brockelhurst (1978) and the continuous line is theoretical for eqn. (3) of the text in which $pK_r = 2.45$, $pK_{el} = pK_{eII} = 3.85$, $k'_{XH} = 850 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k'_{XH_2} = 1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k'_{XH_3} = 1 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k'_{XH_2} = 1 \text{ M}^{-1} \cdot \text{s}^{-1}$.

aspartic acid-158 in papain that has been reported to be an aspartic acid residue (Husain & Lowe, 1970a) may be instead an asparagine residue. Isolation of peptides containing intact asparagine amide groups is notoriously difficult, and residue 64 in papain, now known to be an asparagine residue (Husain & Lowe, 1970a), was originally assigned as an aspartic acid residue (see Drenth *et al.*, 1968). If the residue in ficin is an asparagine residue, an UP-DOWN transition of the type envisaged for papain must be regarded as unlikely, since the $\text{CO}_2^- \cdots \text{HIm}^+$ inter-

action in papain plays a key role in maintaining the DOWN conformation.

(2) The residue in question may be an aspartic acid residue, in accord with the structural evidence, and the carboxy group may possess a pK_a similar to that in papain (pK_a 3–4). If this is the case, it must be assumed that protonation of the carboxylate anion does not lead to a change in the nucleophilic character of the active-centre sulphur atom, and this interpretation also appears to exclude an UP-DOWN transition in ficin of the type envisaged for papain.

(3) The residue in question may be an aspartic acid residue, but the carboxy group may possess a lower pK_a value (say less than 3) than the analogous group in papain. In this case the carboxylate ion would not become protonated to a significant extent in the limited pH range around 4, below which the active-centre thiolate anion itself would become protonated with consequent loss of nucleophilic character. This interpretation preserves the possibility of a pH-dependent UP-DOWN transition in ficin, although it does not require it. If other structural features of the active centre provide a stronger interaction of the carboxylate anion and the imidazolium cation, a relatively low pK_a for the carboxy group might result, but the imidazole group may or may not undergo substantial change in position when it loses its cationic character. The structural features that impose a strong $\text{CO}_2^- \cdots \text{HIm}^+$ interaction might also impose more restricted movement on the histidine side chain even when it is deprotonated. An extreme version of this situation would be a ficin active centre permanently in a conformation somewhat analogous to the DOWN conformation of papain.

The other obvious way in which additional stabilization of an active-centre carboxylate anion in ficin could be achieved would be by the presence of an additional proton-donating (possibly cationic) side chain (e.g. a lysine NH_3^+ group) in close proximity to the aspartate carboxylate anion, with no close analogue in papain. The association of this side chain with the aspartate carboxylate ion either with or without the additional association of the active-centre imidazolium ion could be invoked to provide the required perturbation of the carboxy-group pK_a . If the carboxylate anion is engaged by another cationic site instead of the imidazolium ion, the active centre of ficin may be permanently in a conformation that approximates to the UP conformation of papain. If, however, the carboxylate anion associates with both the imidazolium ion and another cationic site, the possibility of an UP-DOWN transition in neutral and alkaline media is preserved.

Examination of what little structural information on ficin is available provides no evidence for a

cationic side chain close to the active-centre carboxy group additional to that of the active-centre histidine residue (Wong & Liener, 1964; Drenth *et al.*, 1968; Husain & Lowe, 1968*a,b*, 1970*b*; Liener & Friedenson, 1970). Indeed, although there is a lysine residue at position 156 in papain near to aspartic acid-158 in the primary sequence, the analogous residue in ficin is a serine residue. Nevertheless, inspection of a molecular model of papain shows that, even if the active-centre region of ficin is of a closely similar general shape to that of papain, possibilities may exist for the presence of an additional cationic centre close to the putative carboxy group of ficin. The possibility that lysine-156 in papain might affect the reactivity of this enzyme towards cationic probes was pointed out by Malthouse & Brocklehurst (1978), but clearly the influence of this residue is much less marked than that of the cationic site in ficin (see below).

Evidence for a cationic site in ficin with no exact analogue in papain obtained by using alkyl 2-pyridyl disulphide reactivity probes

Since changing the structure of the reactivity probe from that of 2,2'-dipyridyl disulphide to that of *n*-propyl 2-pyridyl disulphide (Shipton & Brocklehurst, 1978) and to that of compound (I) (Brocklehurst *et al.*, 1979*a*) appears to affect markedly the binding of the probe molecule to papain, the reactions of these two alkyl 2-pyridyl disulphide probes with ficin were investigated in anticipation of differential effects that might further illuminate the existence and disposition of proton-binding sites in papain and ficin.

(i) *Reactions of papain and ficin with n-propyl 2-pyridyl disulphide.* Characteristics of the reaction

of papain with *n*-propyl 2-pyridyl disulphide have been presented by Shipton & Brocklehurst (1978).

The pH-*k* profile for the reaction of ficin with *n*-propyl 2-pyridyl disulphide (Fig. 2) is of the same general shape as that reported (Malthouse & Brocklehurst, 1976) for the reaction of ficin with 2,2'-dipyridyl disulphide, i.e. a striking rate maximum in the pH region around 3.5, a rate minimum around pH 6.5 and a rate plateau approached at pH values above 10. The characterizing parameters of this profile (pH-independent rate constants, $\bar{k}$ and apparent pK_a values) are compared with those of pH-*k* profiles for the reactions of papain and ficin with a number of pyridyl disulphides in Table 1, and ratios of the reactivities displayed by both enzymes, in three ionization states, towards pairs of the probe reagents are given in Table 2.

(ii) *Comparison of the reactivities of papain and ficin towards 2,2'-dipyridyl disulphide and n-propyl 2-pyridyl disulphide.* For each of these four reactions, there are three reactive ionization states,

Table 2. *Effect of probe structure on the reactivities of the three protonic states (X, XH and XH₂) of the reactions of the thiol groups of papain and ficin*

The ratios of rate constants were calculated by using the data given in Table 1.

Enzyme	$\frac{\bar{k}_{\text{FPDS}}}{\bar{k}_{\text{2PDS}}}$			$\frac{\bar{k}_{\text{compound I}}}{\bar{k}_{\text{2PDS}}}$		
	XH ₂	XH	X	XH ₂	XH	X
	Papain	3.1	2.8	2.9	—	120
Ficin	0.2	2.8	1.3	3	35	20

Fig. 2. *Dependence on pH of the apparent second-order rate constant (k) for the reaction of the active-centre thiol group of ficin with n-propyl 2-pyridyl disulphide in aqueous media at 25°C at I 0.1*

The points are experimental, and the continuous line is theoretical for an equation of the form shown in eqn. (1) of the text in which the system pK_a values [pK_{XH_2} , pK_{XH}], which are analogous to the molecular pK_a values of eqn. (1), pK_{eI} — pK_{eIII}] are pK_{XH_2} = 3.0, pK_{XH_2} = 3.8, pK_{XH} = 8.6 and the values of the pH-independent rate constants are $\bar{k}_{\text{XH}_2}$ = 24 000 $\text{M}^{-1} \cdot \text{s}^{-1}$, $\bar{k}_{\text{XH}}$ = 1100 $\text{M}^{-1} \cdot \text{s}^{-1}$ and $\bar{k}_{\text{X}}$ = 2900.

designated XH_2 , XH and X to indicate their stoichiometries in protons relative to the state that predominates at pH above 10, the X state.

The reactivity of papain towards n-propyl 2-pyridyl disulphide (k_{PPDS}) is about 3 times that of papain towards 2,2'-dipyridyl disulphide (k_{2PDS}) in all three ionization states (Table 2). Because the intrinsic reactivity of n-propyl 2-pyridyl disulphide towards n-propanethiol is about 10-fold lower than the reactivity of 2,2'-dipyridyl disulphide towards the same low-molecular-weight thiol (T. Stuchbury & K. Brocklehurst, unpublished work), the higher reactivity of n-propyl 2-pyridyl disulphide towards papain is probably best interpreted in terms of an improvement in reagent binding consequent on substitution of one of the 2-pyridyl moieties in the reagent for an n-propyl moiety. Since $k_{\text{PPDS}}/k_{\text{2PDS}}$ is about 3 in all three ionization states of the papain reaction (Table 2), the 30-fold improvement in the binding seems to be pH-independent and unaffected by changes both in the enzyme active centre and in the state of ionization of the reagent unless compensation occurs. For the corresponding reactions of ficin, Table 2 shows that (a) the situation in the XH state is the same as for papain, i.e. $k_{\text{PPDS}}/k_{\text{2PDS}}$ is close to 3, and (b) the situation in the X state is not markedly different from that in papain, although the improvement in the binding to ficin may not be quite as great as for papain. The striking difference between papain and ficin occurs in the XH_2 state, where the ratio $k_{\text{PPDS}}/k_{\text{2PDS}}$ for the ficin reaction is only 0.2 as against 3 for the corresponding state in the papain reaction. Since it is only in the XH_2 state that reaction involves predominantly the cationic form of the probe reagent, it seems probable that the improvement in the binding provided by the substitution of an n-propyl moiety for one of the 2-pyridyl moieties is offset in the case of ficin by a repulsion by the enzyme of the cationic end of the probe reagent. The binding of the probe reagent to the active-centre region must presumably be such that an allowed transition-state geometry can be achieved. In the case of the reaction of a thiolate ion with a disulphide the optimal geometry may be one involving a linear arrangement of the three S atoms (Pappas, 1979). There are two obvious reasons why the binding specifically of a cationic reagent to ficin may not be favoured. Either the ficin active centre may contain a hydrophobic region without analogy in papain or it may contain a cationic site without analogy in papain.

If the explanation lay in ficin possessing a hydrophobic region that repels the cationic pyridine ring of n-propyl 2-pyridyl disulphide, the binding of the unprotonated pyridine ring by ficin might be expected to be favoured. No evidence for this in the form of higher values of $k_{\text{PPDS}}/k_{\text{2PDS}}$ for ficin in the XH and X states of the reaction as compared with

those for the papain reaction is provided by the data in Table 2. Furthermore, assessment of the hydrophobicity of the active centres of papain and ficin by the method involving the reactivity-ratio of a pair of isomeric probes described by Malthouse & Brocklehurst (1980) suggests that the solvent environments in the vicinity of the active-centre thiol groups of the two enzymes may be rather similar.

The alternative explanation, that the ficin active centre may possess a cationic group without exact analogue in papain, seems attractive. Not only does it explain the discrimination by ficin against the n-propyl 2-pyridyl disulphide cation, but it offers an explanation in terms of the low- pK_a carboxy group discussed above for the lack of acid catalysis in the reaction of ficin with 4-chloro-7-nitrobenzofurazan and for the lack of inhibition of the reaction of ficin with 2,2'-dipyridyl disulphide.

It must be presumed that the binding modes to ficin of 2,2'-dipyridyl disulphide and n-propyl 2-pyridyl disulphide differ such that the tighter binding mode adopted by the n-propyl 2-pyridyl reagent necessarily locates the pyridyl ring close to the postulated cationic site that adversely affects this binding mode when the pyridyl ring is protonated. This cationic site could be the active-centre imidazolium ion maintained in a position in the ficin active centre that is different from the position that the analogous cation occupies in the DOWN conformation of the papain active centre, or it could be an additional cationic site in ficin as discussed above.

Evidence for differences in the mobilities or dispositions of the active-centre histidine imidazole groups of papain and ficin with respect to the hydrophobic binding areas of these enzymes

The evidence discussed above, that the ficin active-centre region may contain a cationic site in a position with respect to other active-centre groups that has no close analogue in papain, involves the reactivity of ficin towards the n-propyl 2-pyridyl disulphide cation at pH values 3–4. Although the possibility that the ficin active centre may contain a carboxy group with a low pK_a suggests that a DOWN→UP transition may not occur with the same facility at these low pH values as it does in papain, where the protonations are positively co-operative, the operation of a mechanism involving an UP→DOWN transition essentially similar to that envisaged for papain is not excluded. The low pK_a for the carboxy group may not affect the functioning of the active centre in approximately neutral media, and the behaviour of papain and ficin at pH values around 6.5, where catalytic activity is optimal, may be very similar despite the differences in behaviour at low pH. To evaluate this possibility, the reactivity of the ficin thiol group towards compound (I) and its pH-dependence were investi-

gated. The use of compound (I) as a reactivity probe for papain provided evidence in support of the UP-DOWN transition for this enzyme (Brocklehurst *et al.*, 1979a,c). Whereas for the reactions of papain with 2,2'-dipyridyl disulphide and with n-propyl 2-pyridyl disulphide $\bar{k}_{\text{XH}_2} > \bar{k}_{\text{XH}} < \bar{k}_{\text{X}}$, for the reaction of papain with compound (I) $\bar{k}_{\text{XH}_2} < \bar{k}_{\text{XH}} > \bar{k}_{\text{X}}$ (see Table 1). This dramatic change in the shape of the pH- k profile produces, for the reaction with compound (I), a profile similar to that for $k_{\text{cat}}/K_{\text{m}}$ against pH for catalysis. This may be explained if binding of compound (I) occurs to the minor (UP) conformation of papain in a manner analogous to substrate binding and is followed by relaxation to the DOWN conformation in which the 2-pyridyl leaving group is activated by association with the repositioned imidazolium ion of histidine-159. It seems possible that, even if specific substrates are required to bind to the UP conformation of papain for steric reasons, this restriction may not apply to small reactivity probes such as 2,2'-dipyridyl disulphide and n-propyl dipyridyl disulphide. Unless the probe contains appropriate binding sites therefore [as compound (I) may do], association-activation phenomena (see Brocklehurst *et al.*, 1979a) may not be detected.

Because it was necessary to study the reactions of compound (I) in 13.4% (v/v) acetonitrile for solubility reasons, control experiments were performed to ascertain the effect of this solvent on the reaction of the thiol group of ficin with 2,2'-dipyridyl disulphide. The second-order rate constants for the

reactions with ficin and with compound (I) are shown in Table 3, and the relative reactivities in the different ionization states of the reaction are compared in Table 2.

As with papain (Brocklehurst *et al.*, 1979a), the shape of the pH- k profile for the reaction of ficin with 2,2'-dipyridyl disulphide appears to be maintained in 13.4% acetonitrile in that k increases as the pH is both decreased and increased from around pH 6.5. As for the papain reaction, the main difference between the reaction in purely aqueous media and that in 13.4% acetonitrile is that the latter is slower (except in the XH state), particularly in acidic media.

The striking difference between the shapes of the pH- k profiles for the reactions of compound (I) with papain and ficin respectively is shown by the data in Tables 1 and 3. For papain, the profile for the reaction with compound (I) is markedly different from that for the reaction with 2,2'-dipyridyl disulphide, as discussed above, with very high reactivity in the XH state and lower (or zero) reactivities in the XH₂ and X states. With ficin, however, the profiles for the reactions of the two probe reagents are rather similar in shape, in that k is minimal at pH values around 6.5 and increases at both higher and lower pH values. The values of k at all pH values are larger for the reaction of compound (I) than for the reaction of 2,2'-dipyridyl disulphide, which probably reflects better binding by ficin of the larger, more hydrophobic, probe molecule. In the vicinity of the XH₂ state, however, the

Table 3. Second-order rate constants (k) for the reactions of the active-centre thiol groups of papain and ficin with compound (I) and with 2,2'-dipyridyl disulphide in 13.4% acetonitrile at 25°C at 10.1

Enzyme	Probe reagent	pH	$10^{-3} k \text{ (M}^{-1} \cdot \text{s}^{-1})$	Reference
Ficin	2,2'-Dipyridyl disulphide	3.31	10.45	Present work
		3.95	7.43	
		6.52	0.44	
		7.79	0.50	
		9.86	1.51	
Ficin	Compound (I)	3.31	27.5	
		3.95	17.8	
		5.61	14.8	
		6.52	13.7	
		7.79	10.0	
Papain	2,2'-Dipyridyl disulphide	3.9	4.8	Brocklehurst <i>et al.</i> (1979a)
		6.0	0.5	
		9.3	1.0*	
Papain	Compound (I)	3.2	13.0	
		6.0	52.0	
		8.3	33.0†	

* The pH-dependence of k for this reaction is given by eqn. (1) (see the text), where $\text{p}K_{\text{XH}_3} = 3.47$, $\text{p}K_{\text{XH}_2} = 3.73$, $\text{p}K_{\text{XH}} = 8.8$, $\bar{k}_{\text{XH}_2} = 11\,856 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\bar{k}_{\text{XH}} = 430$ and $\bar{k}_{\text{X}} = 1300$.

† The pH-dependence of k for this reaction is given by $k = \bar{k}/[1 + ([\text{H}^+]/K_1) + (K_{\text{II}}/[\text{H}^+])]$, where the pH-independent rate constant $\bar{k} = 5.2 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\text{p}K_1 = 3.5$ and $\text{p}K_{\text{II}} = 8.8$.

ratio $\bar{k}_{\text{compound (I)}}/\bar{k}_{\text{2PDS}}$ is much smaller than the values of this ratio in the XH and X states (Table 2). This finding appears to support the conclusion drawn from the study of the reaction of ficin with *n*-propyl 2-pyridyl disulphide that alkyl 2-pyridyl disulphides in the protonated state detect a cationic site in the ficin active centre.

The most striking aspect of the reaction of ficin with compound (I) is the lack of the marked rate maximum in approximately neutral media (XH state) observed in the corresponding reaction with papain. This difference in profile shape suggests that, whereas binding of compound (I) to papain results in the association of the 2-pyridyl N atom with the active-centre imidazolium ion, no such juxtaposition occurs when compound (I) binds to ficin. Activation of the reagent in this case is brought about by direct protonation at lower pH values, as is the case in the reactions of both papain and ficin with 2,2'-dipyridyl disulphide and with *n*-propyl 2-pyridyl disulphide. Thus the disposition of the relevant hydrophobic binding areas with respect to the active-centre imidazolium ions must be different in the two enzymes.

Two hydrophobic binding areas in papain that may have analogies in ficin need to be considered. The active centre of papain comprises seven subsites each capable of accommodating a single amino acid residue of a peptide substrate (Schechter & Berger, 1967). Four of these subsites (S_1 – S_4) are located on the *N*-terminal side of the scissile peptide bond of a peptide substrate, and the other three (S'_1 – S'_3) on the *C*-terminal side, the numbering being away from the scissile bond. Both S_2 and S'_1 binding sites appear to be appreciably hydrophobic in nature (Schechter & Berger, 1968; Alecio *et al.*, 1974). The S_2 subsite binds the side chain of an aromatic amino acid residue next but one to that containing the scissile bond in the acyl portion of the substrate, and the S'_1 subsite accommodates the first amino acid residue of the leaving group.

The S'_1 subsite of papain appears to consist of the hydrophobic region near to tryptophan-177 and alanine-136 in papain (see Alecio *et al.*, 1974), and it is noteworthy that this subsite may be modified or indeed even created by the movement of the imidazole group of histidine-159 from the UP to the DOWN conformation.

Two possible explanations for the differences in the properties of the active centres of papain and ficin are suggested by the structural data. If the ficin active centre exists in neutral media in a state very different from the DOWN conformation of papain, binding of compound (I) might not be expected to present the 2-pyridyl N atom to the imidazolium ion. An extreme version of this would be a ficin active centre somewhat analogous to the UP conformation of papain. On the other hand, if the ficin active

centre exists permanently in a conformation analogous to the DOWN conformation of papain, compound (I) may be better able to bind in the S'_1 subsite, which could shield the 2-pyridyl N atom from the imidazolium ion. Both of these explanations point to the possibility of a ficin active centre that may be less mobile than the papain active centre, and raise some doubts as to whether the mechanism of ficin-catalysed hydrolysis involves an UP–DOWN transition analogous to that envisaged for papain.

We thank the Science Research Council for a Research Studentship and a Research Assistantship for J. P. G. M., and Professor E. M. Crook for continued support and encouragement.

References

- Alecio, M. R., Dann, M. L. & Lowe, G. (1974) *Biochem. J.* **141**, 495–501
- Allen, G. & Lowe, G. (1973) *Biochem. J.* **133**, 679–686
- Angelides, K. J. & Fink, A. L. (1978) *Biochemistry* **17**, 2659–2668
- Angelides, K. J. & Fink, A. L. (1979a) *Biochemistry* **18**, 2355–2363
- Angelides, K. J. & Fink, A. L. (1979b) *Biochemistry* **18**, 2363–2369
- Baker, E. N. (1977) *J. Mol. Biol.* **115**, 263–277
- Blow, D. M. (1976) *Acc. Chem. Res.* **9**, 145–152
- Brocklehurst, K. (1974) *Tetrahedron* **30**, 2397–2407
- Brocklehurst, K. (1979) *Biochem. J.* **181**, 775–778
- Brocklehurst, K. & Dixon, H. B. F. (1977) *Biochem. J.* **167**, 859–862
- Brocklehurst, K. & Little, G. (1972) *Biochem. J.* **128**, 471–474
- Brocklehurst, K. & Little, G. (1973) *Biochem. J.* **133**, 67–80
- Brocklehurst, K. & Malthouse, J. P. G. (1978) *Biochem. J.* **175**, 761–764
- Brocklehurst, K., Malthouse, J. P. G. & Shipton, M. (1979a) *Biochem. J.* **183**, 223–231
- Brocklehurst, K., Stuchbury, T. & Malthouse, J. P. G. (1979b) *Biochem. J.* **183**, 233–238
- Brocklehurst, K., Herbert, J. A. L., Norris, R. & Suschitzky, H. (1979c) *Biochem. J.* **183**, 369–373
- Drenth, J., Jansonius, J. N., Koekoek, R., Swen, H. M. & Wolthers, B. G. (1968) *Nature (London)* **218**, 929–932
- Drenth, J., Kalk, K. H. & Swen, H. M. (1976) *Biochemistry* **15**, 3731–3738
- Husain, S. S. & Lowe, G. (1968a) *Biochem. J.* **108**, 855–859
- Husain, S. S. & Lowe, G. (1968b) *Biochem. J.* **108**, 861–866
- Husain, S. S. & Lowe, G. (1970a) *Biochem. J.* **116**, 689–692
- Husain, S. S. & Lowe, G. (1970b) *Biochem. J.* **117**, 333–340
- James, M. N. G. (1980) *Can. J. Biochem.* **58**, 251–271
- Kortt, A. A. & Liu, T. Y. (1973) *Biochemistry* **12**, 338–345
- Liener, I. E. & Friedenson, B. (1970) *Methods Enzymol.* **19**, 261–273

- Lowe, G. (1976) *Tetrahedron* **32**, 291–302
- Malthouse, J. P. G. & Brocklehurst, K. (1976) *Biochem. J.* **159**, 221–234
- Malthouse, J. P. G. & Brocklehurst, K. (1978) *Biochem. Soc. Trans.* **6**, 250–252
- Malthouse, J. P. G. & Brocklehurst, K. (1980) *Biochem. J.* **185**, 217–222
- Matthews, D. A., Alden, R. A., Birktoft, J. J., Freer, S. T. & Kraut, J. (1977) *J. Biol. Chem.* **252**, 8875–8883
- Pappas, J. A. (1979) *J. Chem. Soc. Perkin Trans. II* 67–70
- Petkov, D., Christova, E. & Stoineva, I. (1978) *Biochim. Biophys. Acta* **527**, 131–141
- Schechter, I. & Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157–162
- Schechter, I. & Berger, A. (1968) *Biochem. Biophys. Res. Commun.* **32**, 888–902
- Shipton, M. & Brocklehurst, K. (1978) *Biochem. J.* **171**, 385–401
- Shipton, M., Stuchbury, T. & Brocklehurst, K. (1976) *Biochem. J.* **159**, 235–244
- Stuchbury, T., Shipton, M., Norris, R., Malthouse, J. P. G., Brocklehurst, K., Herbert, J. A. L. & Suchitzky, H. (1975) *Biochem. J.* **151**, 417–432
- Wong, R. C. & Liener, I. E. (1964) *Biochem. Biophys. Res. Commun.* **17**, 470–474