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Characterization of papaya peptidase A as a cysteine proteinase of *Carica papaya* L. with active-centre properties that differ from those of papain by using 2,2′-dipyridyl disulphide and 4-chloro-7-nitrobenzofurazan as reactivity probes

Use of two-protonic-state electrophiles in the identification of catalytic-site thiol groups

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1. The proteinase papaya peptidase A, one of the major components of the latex of *Carica papaya* L., was shown to contain 1 thiol group per molecule; this thiol group is essential for catalytic activity and is part of the catalytic site. 2. The usefulness of two-protonic-state reactivity probes coupled with modification/activity-loss data in assigning a thiol group as an integral part of the catalytic site as against merely 'essential' for activity is discussed. 3. The active centre of papaya peptidase A was investigated by using 2,2′-dipyridyl disulphide and 4-chloro-7-nitrobenzofurazan as reactivity probes. The presence in the enzyme in weakly acidic media of an interactive system containing a nucleophile S atom (pK_I 3.9, pK_{II} 7.9) was demonstrated. 5. Papaya peptidase A resembles ficin (EC 3.4.22.3) and actinidin (the cysteine proteinase from *Actinidin chinensis*) in that it does not appear to possess a carboxy group able to influence the reactivity of the thiol group by change of ionization state at pH values of about 4, a situation that contrasts markedly with that which obtains in papain. 6. Implications of the results for possible variations in cysteine proteinase mechanism are discussed.

Papaya peptidase A is one of the little-studied enzymes present in the dried latex of *Carica papaya* L. (see Brocklehurst *et al.*, 1981*a*). It constitutes about 20% of the soluble latex protein, whereas the much more extensively studied enzyme papain (EC 3.4.22.2) constitutes only about 5%. As part of an investigation of cysteine proteinase mechanism, the reactivity characteristics of the active-centre thiol group of this enzyme towards the two-protonic-state reactivity probe 2,2′-dipyridyl disulphide and towards Nbf-Cl are now reported and compared with those of the thiol groups of papain and ficin (EC 3.4.22.3). The particular value of two-protonic-state reactivity probes in the characterization of active-centre interaction has been discussed previously (Brocklehurst, 1979; Brocklehurst *et al.*, 1979, 1981*a*). Comparative kinetic study of the reactivities of active-centre nucleophiles in a number of related enzymes towards electrophilic reagents whose reactions are sensitive to differences in the arrangement of active-centre side chains promises to allow assessment of the effect of structural variation in the enzyme on active-centre reactivity and on catalytic competence. The highly electrophilic character of pyridyl disulphide probes permits kinetic study of their reactions with active-centre thiol groups over a very wide range of pH, and this may provide an opportunity to detect both changes in active-centre chemistry consequent on change of ionization state and the effects of conformational transitions, one or other of which may occur outside pH ranges in which less highly electrophilic probe reagents react at convenient rates.

Papaya peptidase A is shown to possess the minimal interactive system common to all of the cysteine proteinases studied so far, in which the essential thiolate ion is maintained in acidic media by association of the thiol group with another acid–base system. Although the rate constants for the reactions of the thiol group of its interactive system with 2,2′-dipyridyl disulphide in acidic media are comparable with those of the analogous reaction of

Abbreviations used: Bz-Arg-OEt, α-*N*-benzoyl-L-arginine ethyl ester; Nbf-Cl, 4-chloro-7-nitrobenzofurazan.

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papain and much lower than those of the ficin reaction, the active centres of ficin and papaya peptidase A may be similar in that both appear to lack a carboxy group with characteristics analogous to those of the aspartic acid-158 residue of papain.

Materials and methods

Materials

The latex of *Carica papaya* L. from which papaya peptidase A was prepared was the spray-dried refined product known to have been prepared by the Boudart (1969, 1970) process in Zaire and was kindly supplied by Powell and Scholefield, Liverpool, U.K. α-*N*-Benzoyl-L-arginine ethyl ester hydrochloride (Bz-Arg-OEt) and 4-chloro-7-nitrobenzofurazan (Nbf-Cl) were obtained from Sigma Chemical Co. 2,2′-Dipyridyl disulphide was the Aldrich product, twice recrystallized from light petroleum (b.p. 60–80°C) and as usual had m.p. 58°C. Sulphopropyl-(SP-)Sephadex C-50 and other chromatographic materials were obtained from Pharmacia, London W.5, U.K.

Methods

Papaya peptidase A was isolated from the spray-dried papaya latex by chromatography on SP-Sephadex C-50 as described by Baines & Brocklehurst (1982). Esterase activity was determined titrimetrically by using a Radiometer pH M62 pH-meter, TTA3 titration assembly, ABA13 autoburette, REC 61 Servograph and REA 160 titrograph. Spectral measurements were made with a Cary 118 spectrophotometer, and kinetic runs with probe reagents were performed at 25°C with a Dionex model D110 stopped-flow system coupled to a Tektronix S1 03N storage oscilloscope for reactions of 2,2′-dipyridyl disulphide and with a Cary 118 or Cary 16K spectrophotometer for Nbf-Cl reactions.

Concentrations were determined as follows: papaya peptidase A, by using $\varepsilon_{280} = 4.41 \times 10^4 \text{M}^{-1} \cdot \text{cm}^{-1}$ (calculated from $A^{1\%}_{1\text{cm}} = 18.3$ and M_r 24100, given by Robinson, 1975); 2,2′-dipyridyl disulphide, by using $\varepsilon_{281} = 1.02 \times 10^4 \text{M}^{-1} \cdot \text{cm}^{-1}$ (and occasionally as an additional check by thiolysis with 2-mercaptoethanol; see Brocklehurst & Little, 1973; Shipton & Brocklehurst, 1978); 2-thiopyridone, at pH values below 8.5, by using $\varepsilon_{343} = 8.08 \times 10^3 \text{M}^{-1} \cdot \text{cm}^{-1}$; Nbf-Cl at pH 6.0 in 6.7% (v/v) ethanol by using $\varepsilon_{343} = 1.17 \times 10^4 \text{M}^{-1} \cdot \text{cm}^{-1}$ (Shipton, 1976). Reactions of papaya peptidase A with Nbf-Cl that were monitored at 411 nm produced the *S*-substituted product ($\lambda_{\text{max.}}$ 411 nm, $\varepsilon_{411} = 1.16 \times 10^4 \text{M}^{-1} \cdot \text{cm}^{-1}$).

Most of the other materials and methods, notably buffers and determination of second-order rate constants in reactivity-probe experiments and some aspects of the determination of the characterizing parameters of pH–*k* profiles by an optimization procedure, have been described previously (see Brocklehurst, 1974; Shipton *et al.*, 1976; Brocklehurst & Malthouse, 1980). In the present work the optimization of sigmoidal and bell-shaped components of pH–*k* profiles was performed by using a computer program (MODFIT) and an MU-BASIC PDP 11/10 or PDP 11/23 computer. The MODFIT program makes use of a modified Newton–Raphson method and was found to produce solutions much more quickly than the optimization used previously, which was based on the method of Nelder & Mead (1965) as described by O'Neill (1971).

Results and discussion

Thiol group of papaya peptidase A

This enzyme prepared from refined spray-dried papaya latex was found, in the present work, to contain 0.9–1.1 mol of SH group/mol of protein, as assessed by titration with 2,2′-dipyridyl disulphide at pH 4 and 8 in a conventional spectrophotometer. When the progress curves for the reactions were obtained under pseudo-first-order conditions with 2,2′-dipyridyl disulphide in large excess at about 50 pH values in the range 3.0–10.0, single-phase first-order increases in A_{343} corresponding to reaction of 1 SH group/molecule were obtained. Thus the thiol content and the homogeneity of its reactivity characteristics provide no evidence for the presence of more than 1 accessible SH group/molecule of papaya peptidase A. Evidence that this thiol group is essential for catalytic activity is provided by the results shown in Fig. 1. Reaction of 1 mol of 2,2′-dipyridyl disulphide with 1 mol of fully active papaya peptidase A at pH 4.1 abolished hydrolytic activity towards Bz-Arg-OEt. Addition of excess of 2-mercaptoethanol results in rapid and complete regain of enzyme activity concomitant with release of 1 mol of 2-thiopyridone/mol of protein. Evidence that this 'essential' thiol group is indeed part of the catalytic site of papaya peptidase A is provided by the form of the pH-dependence of the rate of reaction of the thiol group with 2,2′-dipyridyl disulphide (see below).

The usual problem associated with identification of a given amino acid side chain as part of an enzyme catalytic site is that abolition of catalytic activity consequent on specific chemical modification of the side chain is open to a number of interpretations, such as steric shielding of another essential residue or conformational distortion induced by the blocking group. Identification of an 'essential' thiol group of a proteinase that can be shown also to be part of an interactive system by study of its reactivity characteristics towards a two-protonic-state electrophile and application of the

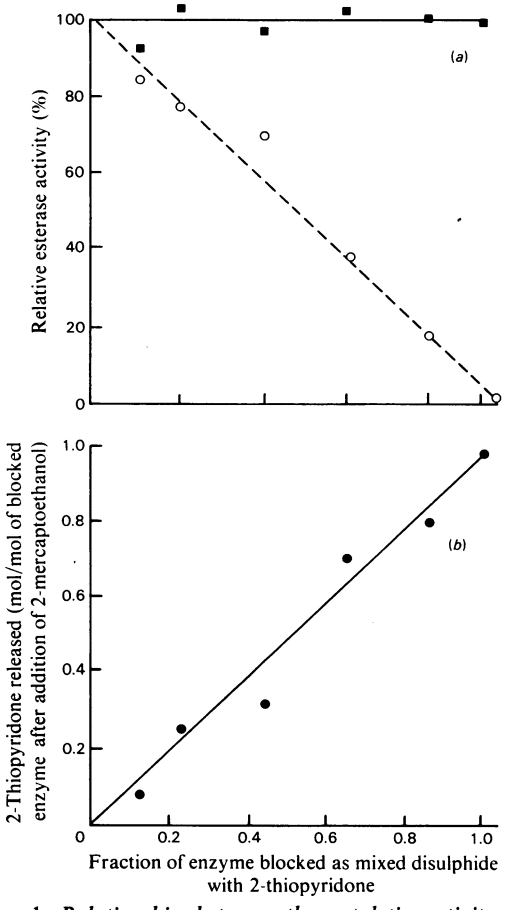

Fig. 1. *Relationship between the catalytic activity of papaya peptidase A and its possession of a free thiol group*

(*a*) Esterase activity towards Bz-Arg-OEt after reaction with various amounts of 2,2'-dipyridyl disulphide: ■, assayed in the presence of 10mM-2-mercaptoethanol; ○, assayed in the absence of 2-mercaptoethanol; activities are expressed as percentages of the activity of the fully active unmodified enzyme. (*b*) Release of 2-thiopyridone (measured by increase in A_{343}) from the papaya peptidase A–2-pyridyl disulphide by treatment with excess of 2-mercaptoethanol.

'three-states criterion' (Brocklehurst, 1974) seems to strengthen greatly the reliability of assignment of the thiol group as an integral part of the catalytic site. The results obtained with two-protonic-state electrophiles in the study of papain (see, e.g., Brocklehurst *et al.*, 1981*a*), ficin (Malthouse & Brocklehurst, 1976), actinidin (Brocklehurst *et al.*, 1981*b*) bromelain (Brocklehurst *et al.*, 1972), thiolsubtilisin (Brocklehurst & Malthouse, 1981) and now in the study of papaya peptidase A suggest that this method of identification of catalytic-site thiol groups should be reliable. The only other methods available at present involve crystallographic analysis or the use of thionoester substrates and the detection of dithioester intermediates by spectral analysis (Lowe & Williams, 1965). Neither of these methods is as convenient as the use of 2,2'-dipyridyl disulphide.

Kinetics of the reaction of the thiol group of papaya peptidase A with 2,2'-dipyridyl disulphide and with Nbf-Cl

Conventional first-order plots of the data from the stopped-flow records of the 2,2'-dipyridyl disulphide reactions were linear up to at least 85% reaction for reactions in the pH range 3.0–10.0, demonstrating that they are first-order with respect to time in enzyme thiol group. At pH values below 3.0, the progress curves were biphasic. For example, at pH 2.32 a rapid increase in A_{343} (second-order rate constant, $k = 4913\,M^{-1}\cdot s^{-1}$) corresponding to reaction of 0.6 of the enzyme thiol group is followed by a much slower reaction corresponding to reaction of 0.4 of enzyme thiol group. This decrease in amplitude at low pH of the rapid reaction with 2,2'-dipyridyl disulphide, which is characteristic of intact active-centre geometry in a number of cysteine proteinases (see, e.g., Brocklehurst, 1979, 1982), has been observed to occur also with papain and ficin. The slow reaction is taken to be due to acid-denatured enzyme molecules with distorted active centres that lack the interaction necessary to generate nucleophilic character in acid media. The residual fast phase is considered to represent reaction of enzyme containing an interactive active-centre system remaining during the sweep time of the stopped-flow experiment. The amplitude of the fast phase decreases progressively as the pH is lowered below 3.

The dependence of the first-order rate constant on the concentration of 2,2'-dipyridyl disulphide (the reagent in excess) was shown to be linear up to 0.75mM at pH 3.59, 6.08 and 8.91 (Fig. 2). This result demonstrates that, in all three pH regions in which reactive protonic states of the reaction predominate (see Fig. 3), any enzyme–reagent adsorptive complexes that might occur on the reaction pathway are not detectable by exhibition of rate saturation, and that it is legitimate to compute second-order rate constants, k, for these reactions by dividing the first-order rate constant obtained from the data in the stopped-flow record by the concentration of 2,2'-dipyridyl disulphide.

The pH-dependence of k is shown in Fig. 3. The pH–k profile displays all of the major features that characterize the reactions of some other cysteine proteinases with the two-protonic-state reactivity probe 2,2'-dipyridyl disulphide, i.e. a striking rate maximum at pH 3–4, a rate minimum at pH about 6–7 and a rate plateau at high pH. The existence of these three reactive protonic states compels the view

that the thiol group of papaya peptidase A interacts with another acid–base system in the enzyme to produce an ionic state in acidic media in which the thiol group possesses nucleophilic character additional to the state at high pH in which the uncomplicated thiolate ion predominates.

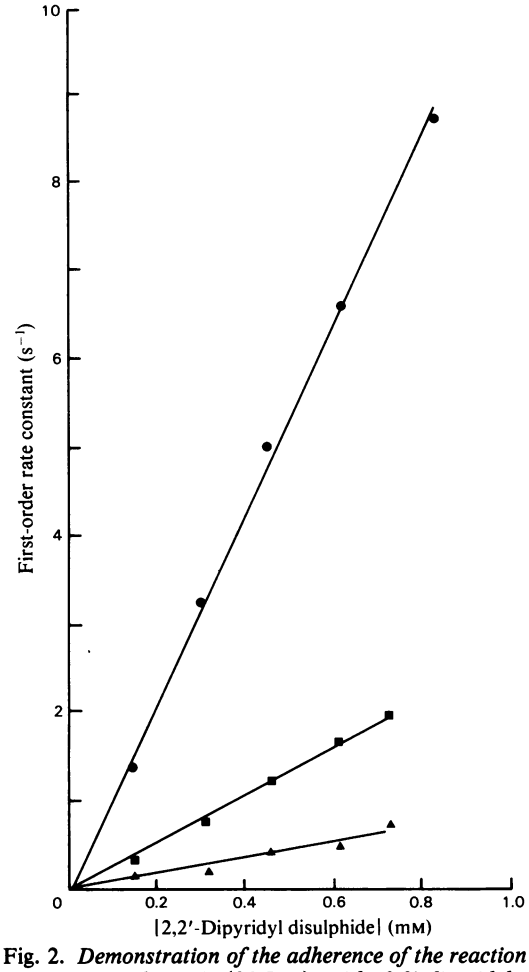

Fig. 2. *Demonstration of the adherence of the reaction of papaya peptidase A (21.5 μM) with 2,2'-dipyridyl disulphide to first-order kinetics with respect to concentration in 2,2'-dipyridyl disulphide at 25°C at I0.1 and at pH3.59 (●), pH6.08 (▲) and pH8.91 (■)*

The increase in k at low pH in the bell-shaped component of the profile in Fig. 3 is interpreted to result from protonation of the probe molecule, which increases its electrophilic reactivity by a factor of at least 1×10^3 (see Brocklehurst *et al.*, 1981*a*). The most effective reaction in the ionization state around the pH optimum seems most likely to be that of the S atom of the interactive system of the active centre with the 2,2'-dipyridyl disulphide monocation, and it is not necessary for the pK_a of the reagent to be exhibited as a major feature of the pH–k profile for this to be true, as was discussed in connection with the analogous reaction of papain (Brocklehurst & Malthouse, 1980).

The pH-dependences of k for the reactions of 2,2'-dipyridyl disulphide with papaya peptidase A, papain and ficin are compared in Fig. 3, and the characterizing parameters of the pH–k profiles (pH-independent rate constants, $\tilde{k}$ and pK_a values) are given in Table 1. Despite the similarity of the overall shapes of the three pH–k profiles of Fig. 3, there are substantial differences in the bell-shaped components at low pH (the XH_2 states). Although the bell-shaped components of the profiles for papaya peptidase A and papain appear to be rather similar in the magnitudes of the k values, with the profile for papaya peptidase A displaced somewhat to lower pH values, this may be deceptive. Only the papain reaction appears to display positively co-operative protonic dissociations with $pK_{II} - pK_I < 0.6$ (half-height width <1.53) (see Dixon, 1973). The papaya peptidase A bell has a half-height width of 1.8 and $pK_{II} - pK_I = 1.12$, which resembles more the shape of the ficin bell (half-height width, 1.8; $pK_{II} - pK_I - 1.38$), and, like the ficin profile, that for the papaya peptidase A reaction has pK_I (2.76) reasonably close to the pK_a value of the 2,2'-dipyridyl disulphide monocation (2.45).

For the papaya peptidase A reaction, however, the discrepancy, $2.76 - 2.45 = 0.31$, is sufficient to raise doubts as to whether pK_I reflects only deprotonation of the cationic form of the probe reagent. It seems unlikely, however, that deprotonation of the probe reagent would not affect the reaction rate in the pH region 2–3, and the unit slope of the log k–pH plot (not shown) in this pH region

Table 1. *Characterizing parameters of the reactions of 2,2'-dipyridyl disulphide with papaya peptidase A, papain and ficin at 25°C at I0.1*

The characterizing parameters $pK_I - pK_{III}$ and pH-independent rate constants $\tilde{k}_1 - \tilde{k}_3$ relate to the pH–k profiles shown in Fig. 3.

	pK_I	pK_{II}	pK_{III}	$10^{-4}\,\tilde{k}_1$ ($M^{-1} \cdot s^{-1}$)	$10^{-2}\,\tilde{k}_2$ ($M^{-1} \cdot s^{-1}$)	$10^{-3}\,\tilde{k}_3$ ($M^{-1} \cdot s^{-1}$)	Reference
Papaya peptidase A	2.76	3.88	7.92	1.85	5.0	3.3	Present work
Papain	3.85	3.90	8.75	4.2	8.5	1.7	Shipton & Brocklehurst (1978)
Ficin	2.42	3.82	8.61	11.1	4.0	2.2	Malthouse & Brocklehurst (1976)

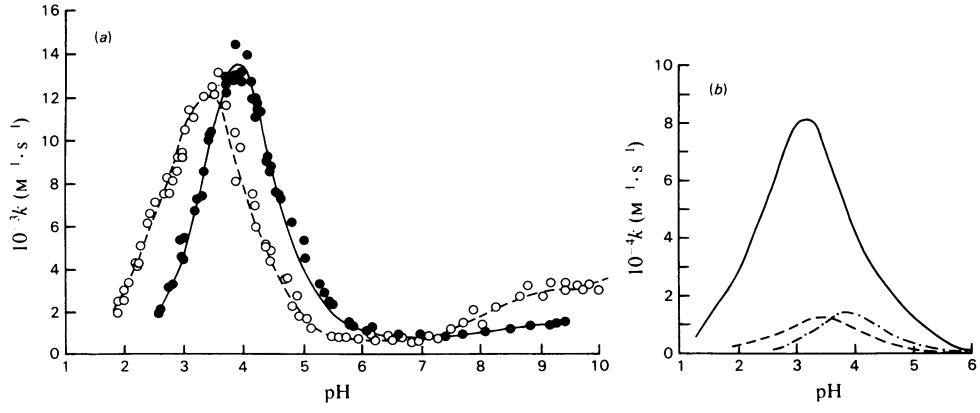

Fig. 3. *Dependence on pH of the apparent second-order rate constant (k) for the reaction of papaya peptidase A with 2,2'-dipyridyl disulphide at 25°C at I0.1 and comparison with the pH-dependences of the analogous reactions of papain and ficin*

(*a*) Experimental results for the papaya peptidase A reaction (○) and the papain reaction (●). (*b*) Fitted lines showing the pH-dependences for the analogous reactions of papaya peptidase A, papain and ficin. In both (*a*) and (*b*) the lines are theoretical for:

$$k = \frac{\bar{k}_1}{\left(1+\frac{[H^+]}{K_I}+\frac{K_{II}}{[H^+]}\right)} + \frac{\bar{k}_2}{\left(1+\frac{[H^+]^2}{K_I K_{II}}+\frac{[H^+]}{K_{II}}+\frac{K_{III}}{[H^+]}\right)} + \frac{\bar{k}_3}{\left(1+\frac{[H^+]}{K_{III}}\right)}$$

where, for papaya peptidase A (----), $\bar{k} = 1.85\times10^4\,M^{-1}\cdot s^{-1}$, $\bar{k}_2 = 500\,M^{-1}\cdot s^{-1}$; $\bar{k}_3 = 3.30\times10^3\,M^{-1}\cdot s^{-1}$; $pK_I = 2.76$, $pK_{II} = 3.88$, $pK_{III} = 7.92$; for papain (Shipton & Brocklehurst, 1978) [—— in (*a*), -·-·- in (*b*)], $\bar{k} = 4.2\times10^4\,M^{-1}\cdot s^{-1}$, $\bar{k}_2 = 850\,M^{-1}\cdot s^{-1}$, $\bar{k}_3 = 1.7\times10^3\,M^{-1}\cdot s^{-1}$, $pK_I = 3.85$, $pK_{II} = 3.90$, $pK_{III} = 8.8$; for ficin (Malthouse & Brocklehurst, 1976) (——), $\bar{k}_1 = 1.11\times10^5\,M^{-1}\cdot s^{-1}$, $\bar{k}_2 = 400\,M^{-1}\cdot s^{-1}$, $\bar{k}_3 = 2.23\times10^3\,M^{-1}\cdot s^{-1}$, $pK_I = 2.42$, $pK_{II} = 3.82$, $pK_{III} = 8.61$.

provides no evidence for the influence of a second ionizing group on the reaction rate. It is possible, however, that enhanced reactivity of the 2,2'-dipyridyl disulphide dication might provide a plateau rate at very low pH, and this might account for the high value (by 0.3 unit) of pK_I.

The other striking piece of evidence for two pK_a values each close to 4 in the papain active centre was obtained by using Nbf-Cl as a reactivity probe. The bell-shaped component for the reaction in acidic media characterized by two apparently co-operative pK_a values close to 4 (Shipton *et al.*, 1976) appears to support the conclusions about the papain active centre suggested by the results obtained with the 2,2'-dipyridyl disulphide probe (Fig. 3). In the Nbf-Cl reaction the undissociated form of the carboxy group of the aspartic acid-158 residue of papain appears to assist the reaction (Shipton *et al.*, 1976), whereas in the 2,2'-dipyridyl disulphide reaction protonation of the carboxylate anion could inhibit the reaction (Brocklehurst & Malthouse, 1980).

Kinetic study of the reaction of Nbf-Cl with papaya peptidase A in the pH range approx. 3–5 (Table 2) shows that, as was found for the analogous ficin reaction and in marked contrast with the papain reaction, there is no rate acceleration caused by protonation of the enzyme.

Table 2. *pH-dependence of the reactions of the thiol groups of papaya peptidase A, papain and ficin with Nbf-Cl at 25°C at I0.1*

Enzyme	pH	k ($M^{-1}\cdot s^{-1}$)	Reference
Papaya peptidase A	3.1	<1	Present work
	3.5	1.2	
	4.1	5.0	
	4.25	11.2	
	4.7	38.5	
Papain	2.65	15.0	Shipton *et al.* (1976)
	3.2	28.6	
	3.4	32.5	
	4.1	17.6	
	4.4	8.5	
Ficin	3.25	0.08	
	3.7	0.18	
	4.3	0.27	
	4.7	0.33	

Conclusions

Results obtained with 2,2'-dipyridyl disulphide as a two-protonic-state reactivity probe and applica-

tion of the three-state criterion (Brocklehurst, 1974) strongly suggest the presence of an interactive system in the active centre of papaya peptidase A in which a nucleophilic S atom is maintained by association with another acid–base system, presumably, by analogy with papain and ficin, a histidine imidazole side chain. Whether the reactivity of this interactive diad is further modulated by association with a carboxylate ion analogous to that of the aspartic acid-158 residue of papain is less certain. Neither the reaction of 2,2′-dipyridyl disulphide nor the reaction of Nbf-Cl reveals the influence of a second ionizing group in the enzyme on the thiol-group reactivity. It seems likely that, if a carboxy group is an integral part of the catalytic site of papaya peptidase A, its pK_a is outside the range in which change of ionization state could influence the reactions of the thiol group with these probe reagents. This conclusion should apply also to ficin, and papain appears to be the only cysteine proteinase studied to date in which two positively co-operative ionizations with pK_a values close to 4 control the reactivity of the active-centre thiol group. Positive co-operativity in proton binding presumably demands a suitable conformational change. In the case of papain, the movement of the imidazolium ion of the postulated $S^-/ImH^+/CO_2^-$ triad (see Angelides & Fink, 1978, 1979*a*,*b*) consequent on protonation of one of the anionic sites (thiolate or carboxylate) could facilitate protonation of the other anionic site, and to this extent the co-operative ionizations displayed in these probe reactions appear to support the UP/DOWN model proposed by Angelides & Fink (1978). It remains to be discovered whether any other cysteine proteinase displays these reactivity characteristics and whether those that do not, like papaya peptidase A and ficin, also function by means of a two-state mechanism, possibly involving a carboxy group with a much lower pK_a than that of the papain carboxy group, or by a one-state mechanism, as appears to be the case for actinidin.

The cysteine proteinase actinidin was shown previously (Brocklehurst *et al.*, 1981*b*) to resemble ficin rather than papain in a number of respects, including the inability of its known active-centre carboxy group to influence thiol-group reactivity at pH values of about 4. In actinidin the active centre is known to predominate at least in the crystal in the UP conformation, where interaction with the carboxy group would not be predicted. The value of pK_I of the active centre, which controls production of the interactive nucleophilic system, determined by using n-propyl 2-pyridyl disulphide as a reactivity probe (Brocklehurst *et al.*, 1981*b*), is 3.1, appreciably lower than the pK_I values of papain, ficin or papaya peptidase A, all of which are 3.8–3.9, and this might reflect a different type of interactive system in which the S^-/ImH^+ associated is not 'loosened' by further association of ImH^+ with CO_2^-. The question whether there are two interactive systems in papain, one an S^-/ImH^+–SH/Im diad in the UP conformation and the other an $S^-/ImH^+/CO_2^-$ triad, is yet to be answered. It is noteworthy, however, that the interpretation of the Nbf-Cl probe data as presently perceived appears to require an interactive diad, which could be in the UP conformation, with the carboxy group free to activate the nitrooxadiazole reagent. Somewhat paradoxically, only papain, of the cysteine proteinases investigated so far, exhibits optimal activity towards Nbf-Cl, and actinidin, the only cysteine proteinase for which structural evidence obtained in weakly acidic media appears to rule out a DOWN conformation for the active centre, does not. Although the co-operative protonic dissociation exhibited by papain in the 2,2′-dipyridyl disulphide reaction could reflect the DOWN→UP transition, it is difficult to see how a similar interpretation could hold for the Nbf-Cl reaction, in which reaction of an UP conformation interactive system appears to be required. If the apparently co-operative ionizations reflected in both 2,2′-dipyridyl disulphide and Nbf-Cl reactions with papain have common cause, this would seem to weaken the evidence for a two-state mechanism for papain. The only evidence that would then remain strongly in favour of such a mechanism is the free-enzyme isomerization detected by Angelides & Fink (1979*b*) by cryokinetic methods with benzyloxycarbonyl-lysine *p*-nitroanilide as substrate. Clearly much remains to be discovered about cysteine proteinase active-centre structure and mechanism, and it would be helpful to ascertain at an early stage whether a two-state mechanism does operate in papain, whether papain is unique in this respect, and whether actinidin is unique in operating via a one-state (UP conformation) mechanism with a particularly low pK_I for the interactive system.

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