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Differences in the interactions of the catalytic groups of the active centres of actinidin and papain

Rapid purification of fully active actinidin by covalent chromatography and characterization of its active centre by use of two-protonic-state reactivity probes

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1. A rapid method of isolation of fully active actinidin, the cysteine proteinase from *Actinidia chinensis* (Chinese gooseberry or kiwifruit), by covalent chromatography, was devised. 2. The active centre of actinidin was investigated by using n-propyl 2-pyridyl disulphide, 4-(*N*-aminoethyl 2'-pyridyl disulphide)-7-nitrobenzo-2-oxa-1,3-diazole and 4-chloro-7-nitrobenzofurazan as reactivity probes. 3. The presence in actinidin in weakly acidic media of an interactive system containing a nucleophilic sulphur atom was demonstrated. 4. The pK_a values (3.1 and 9.6) that characterize this interactive system are more widely separated than those that characterize the interactive active-centre systems of ficin (EC 3.4.22.3) and papain (EC 3.4.22.2) (3.8 and 8.6, and 3.9 and 8.8 respectively). 5. Actinidin was shown to resemble ficin rather than papain in (i) the disposition of the active-centre imidazole group with respect to hydrophobic binding areas, and (ii) the inability of the active-centre aspartic acid carboxy group to influence the reactivity of the active-centre thiol group at pH values of about 4. 6. The implications of the results for one-state and two-state mechanisms for cysteine-proteinase catalysis are discussed.

The study of the cysteine proteinases as a group of analogous enzymes promises to allow the evaluation of the effect of structural variation in active centres on catalytic competence (Brocklehurst *et al.*, 1980; Brocklehurst & Malthouse, 1980, 1981). There is particular interest at present in actinidin, the cysteine proteinase from the fruit of the Chinese gooseberry or kiwifruit (*Actinidia chinensis*), because its structure at 0.17nm has recently been described (Baker, 1980). The only other cysteine proteinase whose structure has been determined (this structure at 0.28nm) is papain (EC 3.4.22.2) [see Brocklehurst *et al.* (1981) for a review].

Abbreviations used: Py-S-S-Pr, n-propyl 2-pyridyl disulphide; Nbf-Cl, 4-chloro-7-nitrobenzofurazan; Cbz-Lys-ONp, *N*-α-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester; Im, imidazole; ImH^+, imidazolium cation.

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A central problem of cysteine-proteinase mechanism concerns the way in which essential proton transfers are effected and coupled to other bond-making and bond-breaking steps to permit facile acylation by substrate of the active-centre thiol group. The mobility of the active-centre histidine imidazole group, its disposition with respect to relevant hydrophobic binding areas and the existence and conformation of a residue analogous to aspartic acid-158 in papain are worthy of special attention in this connection. Techniques for the study of these facets of cysteine-proteinase mechanism involving the use of alkyl 2-pyridyl disulphides as two-protonic-state reactivity probes have been discussed by Brocklehurst & Malthouse (1980) and used to characterize notable differences between the active centres of papain and ficin (EC 3.4.22.3).

In the present paper, a kinetic study of the reactions of the active-centre thiol group of actinidin (that of cysteine-25) with the two-protonic-state reactivity probes n-propyl 2-pyridyl disulphide

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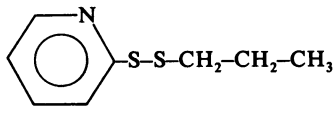
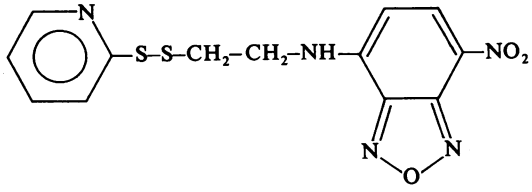


(I)



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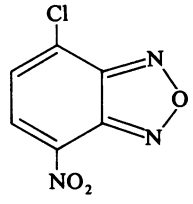

(III)

(Py-S-S-Pr) (I) and 4-(*N*-2-aminoethyl 2′-pyridyl disulphide)-7-nitrobenzo-2-oxa-1,3-diazole (II) and with 4-chloro-7-nitrobenzofurazan (Nbf-Cl) (III) is reported.

The results demonstrate the existence in actinidin in weakly acidic media of an interactive system containing a nucleophilic sulphur atom and mechanistically significant differences between the active centres of actinidin and papain. In addition, a rapid method of isolating fully active actinidin by covalent chromatography is reported.

Materials and methods

Materials

Fresh Chinese gooseberries were obtained from the New Covent Garden Market, Nine Elms, London S.W.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 mincer and the pulp was stored in freezer bags in the deep freeze until required. Py-S-S-Pr (compound I) (Shipton & Brocklehurst, 1978), and 4-(*N*-2-aminoethyl 2′-pyridyl disulphide)-7-nitrobenzo-2-oxa-1,3-diazole (compound II) (Stuchbury *et al.*, 1975) were synthesized as described previously. Nbf-Cl (Aldrich) was purified and stored as described by Shipton *et al.* (1976). The activated Sepharose 6B (2-pyridyl disulphide derivative of mercaptohydroxypropyl ether agarose gel) used for covalent chromatography, which was the product of Pharmacia, was a gift from Dr. J. Carlsson, Pharmacia Diagnostics AB, Uppsala 1, Sweden. Cbz-Lys-ONp was obtained from Sigma.

Isolation of actinidin by covalent chromatography

Frozen Chinese-gooseberry pulp (1 kg) was allowed to thaw at room temperature (approx. 22°C) and was then extracted with 500 ml of 200 mM-L-cysteine, pH 5.0, containing 1 mM-EDTA, by stirring the mixture at room temperature for 1 h. The resulting suspension was filtered through fine-mesh cloth and the cloudy filtrate was centrifuged at 20000 *g* for 30 min at 4°C. The precipitate was discarded and the supernatant was made 1 mM in dithiothreitol. The solution was stirred at room temperature for 10 min and was then adjusted to 60% saturation with $(NH_4)_2SO_4$ by gradual addition of the solid salt (390 g/litre). The resulting suspension was left at 4°C for 30 min and was then centrifuged as described above. The supernatant was discarded and the precipitate was suspended in 300 ml of 0.1 M-sodium acetate buffer, pH 4.4, containing 1 mM-EDTA and 55% saturated with $(NH_4)_2SO_4$ (351 g/litre). The suspension was stirred for 10 min and was then centrifuged as described above. The supernatant was discarded and the precipitate was dissolved in 300 ml of 0.1 M-sodium acetate buffer, pH 4.4, containing 1 mM-EDTA and 0.3 M-NaCl. The small amount of solid that failed to dissolve was removed by centrifugation.

The solution was mixed with 50 ml of activated Sepharose 6B (the gel containing alkyl 2-pyridyl disulphide sites, 15 g dry weight) that had been allowed to equilibrate in the acetate buffer, and the mixture was stirred at room temperature for 45 min. The gel was isolated on a sintered-glass filter and washed with acetate buffer (approx. 2 litres) until the values of A_{343} and A_{280} of the filtrate had each fallen to less than 0.03. The gel was allowed to equilibrate in 2 litres of 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.3 M-NaCl and 1 mM-EDTA, on the sintered filter and was then packed into a column (40 cm × 2.5 cm). The covalently bound actinidin was released from the gel by elution with a mixture of 20 mM-L-cysteine and 1 mM-dithiothreitol in the Tris/HCl buffer by using an LKB 7000 Ultrarac fraction collector (10 ml fractions) and a flow rate of 60 ml/h. A_{280} was recorded during the elution and the fractions were analysed by measurement of catalytic activity towards Cbz-Lys-ONp. Those fractions with $A_{280} \geqslant 2$ were pooled and adjusted to 50% saturation with $(NH_4)_2SO_4$ (313 g/litre). The resulting suspension was stored at 4°C for at least 1 h. The precipitate was isolated by centrifugation at 20000 *g* for 1 h at 4°C and stored at 4°C as a suspension in a minimal volume of 0.1 M-KH_2PO_4/NaOH buffer, pH 6.0, containing 1 mM-EDTA and $(NH_4)_2SO_4$ (50% satn.) until required. When stored

in this state, no loss of catalytic activity was apparent during 2 months.

When actinidin was required for kinetic study it was activated and freed from activator as follows: 0.2ml of the suspension was dissolved in 0.2ml of water and 0.4ml of 40mM-L-cysteine in 0.2M-Tris/HCl buffer, pH8.0, at room temperature, and the solution was left at room temperature for 15min. Immediately before separation from low-molecular-weight material on a column (30cm × 5cm) of Sephadex G-25, the solution was adjusted to 1mM-dithiothreitol. The fully active enzyme was eluted by using 0.1M-KCl containing 1mM-EDTA and emerged mainly in a 5ml volume after about 30ml of eluate.

Chromatography on DEAE-cellulose

Samples of actinidin prepared by covalent chromatography were evaluated by ion-exchange chromatography essentially as described by Boland & Hardman (1972).

Kinetic measurements

The specific activity of actinidin towards Cbz-Lys-ONp (0.1mM) was determined at 25°C in 0.1M-phosphate buffer, pH6.0, containing 1.6% (v/v) acetonitrile and 1mM-dithiothreitol. The stock solution of substrate was in acetonitrile/water (19:1, v/v). The release of *p*-nitrophenol was monitored at 348nm by using a Cary 118C spectrophotometer and the rate (corrected for spontaneous hydrolysis of the substrate measured before addition of enzyme) was calculated by using $\Delta\varepsilon_{348} = 5400\,M^{-1}\cdot cm^{-1}$ (Boland & Hardman, 1972). The Michaelis parameters for the catalysis at pH6.0 were determined in the same way, except that the initial substrate concentration was varied in the range 10–230μM and dithiothreitol was omitted from assays in which fully active enzyme (activator-free) was used.

Other materials and methods, notably spectral characteristics of reactivity probes and their thiolysis products, buffers and determination of second-order rate constants (k) at 25°C (I 0.1) and of the characterization parameters of pH–k profiles by an optimization procedure, have been described previously [for reactions of Nbf-Cl, see Shipton *et al.* (1976), and for reactions of other probes, see Brocklehurst & Malthouse (1980) and references therein].

Results and discussion

Isolation of actinidin by covalent chromatography

Isolation of actinidin in other laboratories (Carne & Moore, 1978; McDowall, 1970; Boland & Hardman, 1972; Baker, 1980; Baker *et al.*, 1980) has involved extraction of the pulped fruit, $(NH_4)_2SO_4$ precipitation, dialysis, ion-exchange chromatography, concentration by ultrafiltration, and sometimes crystallization. In our hands, application of this type of method to frozen Chinese-gooseberry pulp required 5–6 days and produced enzyme that was 50–70% active before rechromatography on DEAE-cellulose as described by Boland & Hardman (1972).

By using covalent chromatography, developed for the specific isolation of thiol-containing proteins [Brocklehurst *et al.* (1973, 1974); see also Brocklehurst (1979) and Hillson (1981)], it was possible to produce fully active actinidin containing 1 thiol group with high reactivity towards 2-pyridyl disulphides per molecule of protein in 2 days (yield 450mg of protein/kg of fruit pulp; specific activity towards 0.1mM-Cbz-Lys-ONp at 25°C, pH6.0, I 0.1: 60 units $\cdot$ mg^{-1}). By using covalent chromatography at pH4–5, it is possible to discriminate in favour of cysteine proteinases such as actinidin, even when the solution applied to the chromatography column contains other thiol-containing molecules, including proteins, because of the abnormally high reactivity of the active-centre thiol groups towards the protonated 2-pyridyl mixed-disulphide sites on the gel. This abnormally high reactivity arises from active-centre interaction of a type that is absent in many other thiol-containing molecules (see below). The solution of actinidin produced by covalent chromatography is sufficiently concentrated for the enzyme to be isolated by precipitation with $(NH_4)_2SO_4$ without the ultrafiltration step that was necessary when the ion-exchange method was used. Because the yield of actinidin from Chinese gooseberry is only about 450mg/kg of fruit pulp, compared with a yield of papain from papaya (*Carica papaya*) latex of about 40g/kg of latex, the batchwise process related to covalent chromatography, developed for papain (Stuchbury *et al.*, 1975) cannot be used for the preparation of actinidin. It is necessary to use the original column technique (Brocklehurst *et al.*, 1973) for elution of enzyme from the gel, although it is convenient to use a batchwise process for the preparation of the Sepharose–actinidin conjugate, which is subsequently packed into a column for the elution step.

Catalytic activity of actinidin prepared by covalent chromatography

Actinidin prepared by covalent chromatography contains 1 thiol group per molecule which has high reactivity towards 2,2'-dipyridyl disulphide and towards n-propyl 2-pyridyl disulphide at pH4.5. This high reactivity in acidic media, which results in essentially instantaneous release of the 2-thiopyridone chromophore from the disulphide titrant stoichiometric with enzyme thiol, is charac-

teristic of intact active centres in some other cysteine proteinases (see, e.g., Brocklehurst, 1979, 1981; Brocklehurst *et al.*, 1981). The catalysis of the hydrolysis of Cbz-Lys-ONp by actinidin thus prepared obeys Michaelis–Menten kinetics at pH 6.0 over the range of substrate concentration 10–230 μM, as shown by the linearity of plots of s/v against s. This type of plotting and also computer-fitting of the s, v data to the hyperbolic form of the Michaelis–Menten equation (Cleland, 1963) provided values of $k_{cat.}$ (the catalytic-centre activity = $33 \pm 2\,s^{-1}$ and $K_m = 26 + 2\,\mu M$ (and thus of $k_{cat.}/K_m = 1.3 \times 10^6\,M^{-1} \cdot s^{-1}$). These compare well with the values obtained by Boland & Hardman (1972) for actinidin purified from their initial preparation by rechromatography on DEAE-cellulose ($k_{cat.} = 29\,s^{-1}$; $K_m = 22\,\mu M$ and thus $k_{cat.}/K_m = 1.3 \times 10^6\,M^{-1} \cdot s^{-1}$). Actinidin prepared by covalent chromatography does not contain the inactive protein that is revealed in the preparation obtained by the ion-exchange method as a faster-running component on rechromatography on DEAE-cellulose. The values of the Michaelis parameters characteristic of the catalysis by fully active actinidin prepared by covalent chromatography correspond to a value of the specific activity for 0.1 mM-substrate of 60 units $\cdot$ mg^{-1}, which is similar to that (55 units $\cdot$ mg^{-1}) calculated by substituting the values of $k_{cat.}$ and K_m reported by Boland & Hardman (1972) into the Michaelis–Menten equation.

Demonstration of an interactive system in the active centre of actinidin

The catalytic device employed by the cysteine proteinase papain to catalyse the hydrolysis of amide bonds appears to involve the sulphur atom of cysteine-25 maintained in a highly nucleophilic state in acidic media where the imidazolium ion of histidine-159 can complete the acylation process by general acid catalysis [see Brocklehurst *et al.* (1981) for a review]. That a similar molecular feature exists also in the active centre of actinidin was demonstrated in the present work by studying the kinetics of the reaction of the sulphur atom of cysteine-25 of actinidin with the two-protonic-state reactivity probe Py-S-S-Pr. The basis of the detection of this type of interaction was discussed by Brocklehurst *et al.* (1979) (see also Brocklehurst, 1974; Shipton & Brocklehurst, 1978). Observation of chemical reactivity in three ionization states as in Fig. 1 demonstrates the existence of two nucleophilic states in the enzyme. The ionization state that predominates at high pH (pH > 10.5, the X-state) contains the uncomplicated thiolate ion of cysteine-25 of actinidin. The state that predominates in neutral and weakly acidic media (the XH-state) contains the second nucleophilic state of the enzyme, the

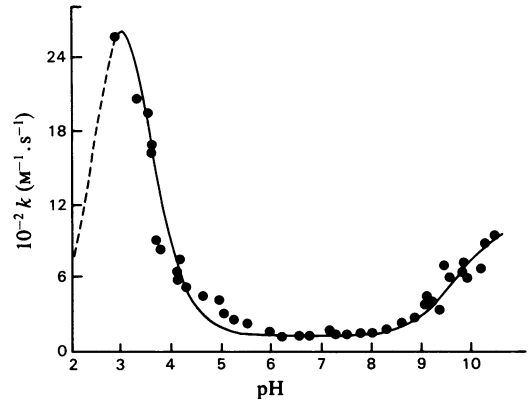

Fig. 1. *Dependence on pH of the apparent second-order rate constant for the reaction of actinidin with Py-S-S-Pr in aqueous media at 25°C, I 0.1*

The points are experimental and the continuous line (and the broken line beyond the data points) is theoretical for:

$$k = \left(\frac{\bar{k}_{XH_2}}{1 + \frac{[H^+]}{K_{XH_3}} + \frac{K_{XH_2}}{[H^+]}} \right) + \left(\frac{\bar{k}_{XH}}{1 + \frac{[H^+]^2}{K_{XH_3} \cdot K_{XH_2}} + \frac{[H^+]}{K_{XH_2}} + \frac{K_{XH}}{[H^+]}} \right) + \left(\frac{\bar{k}_X}{1 + \frac{[H^+]}{K_{XH}}} \right)$$

(see Shipton & Brocklehurst, 1978) and the following values of the parameters: pK_{XH_3} chosen as 2.83, the pK_a of the Py-S-S-Pr cation; $pK_{XH_2} = 3.13$; $pK_{XH} = 9.62$; $\bar{k}_{XH_2} = 6157\,M^{-1} \cdot s^{-1}$; $\bar{k}_{XH} = 125\,M^{-1} \cdot s^{-1}$; $\bar{k}_X = 1057\,M^{-1} \cdot s^{-1}$.

catalytically competent state, which arises from interaction of the thiol group with some other active-centre residue (presumably the side chain of histidine-162). Protonation of the probe molecule at lower pH values increases the rate of its reaction with the interactive system of the enzyme, and this gives rise to the XH_2 state at pH values of approx. 3. The conformational instability of actinidin prevented kinetic analysis at pH values less than 3. At low pH values the stopped-flow record consists of a progress curve that rapidly becomes concave-upwards, which is characteristic of precipitation, and a first-order increase in absorbance of active-centre stoichiometry is no longer obtained. The characterizing parameters (pH-independent rate constants, $\bar{k}$ and molecular or system pK_a values) of Fig. 1 are presented in Table 1. The data collected below pH 4 fit a conventional diprotic model with $pK_I \leqslant 3.0$. If pK_I is assumed to be 2.83, the pK_a value of Py-S-S-Pr (Shipton & Brocklehurst, 1978) the values of the $\bar{k}_{XH_2}$ and pK_{II} are those given in Table 1.

Table 1. *Characteristics of the reactions of the active-centre thiol groups of actinidin, ficin and papain with Py-S-S-Pr each in three protonic states (X, XH and XH_2) in aqueous solution at 25°C and I 0.1*

For the reaction of actinidin, the parameters correspond to the theoretical curve of Fig. 1; the value pK_{XH_3} is assumed to be that of the pK_a value of the monocation of Py-S-S-Pr.

	Molecular pK_a			pH-independent rate constant ($M^{-1} \cdot s^{-1}$)			
Enzyme	pK_{XH_3}	pK_{XH_2}	pK_{XH}	k_{XH_2}	k_{XH}	k_X	Reference
Actinidin	2.83	3.13	9.62	6157	125	1057	The present work
Ficin	3.0	3.8	8.6	24000	1100	2900	Brocklehurst & Malthouse (1980)
Papain	3.86	3.89	8.8	131000	2400	5000	Shipton & Brocklehurst (1978)

Comparison of the active centres of actinidin, papain and ficin

(*i*) *Reactivities towards Py-S-S-Pr.* All three active centres display nucleophilic character in two ionization states, which gives rise to the three reactive ionization states XH_2, XH and X (see Table 1). In all three states the order of reactivity is papain > ficin > actinidin. The reactivity of actinidin is particularly low, relative to the other two enzymes, in the XH state, which would be expected to characterize mainly the reaction of the catalytically competent form of the enzyme with the neutral probe molecule. Thus the reactivity of actinidin in this state is 9 times less than that of ficin and 19 times less than that of papain. The relationship between the reactivities of actinidin and papain towards Py-S-S-Pr is somewhat similar to that between their catalytic activities towards hydrophobic substrates, the value of $k_{cat.}/K_m$ for papain being about 10–100 times greater than that for the analogous actinidin catalysis. Although differences in binding could account in part for these differences, both for the substrates (Baker *et al.*, 1980) and even for small probe reagents that are not close substrate analogues, the differences in the active-centre pK_a values (Table 1) suggest that the intrinsic reactivity of the interactive system of actinidin might be different from those of papain and ficin. For papain and ficin, the molecular pK_a values that characterize the formation of the interactive system from SH and ImH^+ and its decomposition to S^- and Im are 3.8–3.9 for formation and 8.6–8.8 for decomposition, i.e. the two pK_a values are separated by about 5 pK_a units. For actinidin, however, the pK_a for the formation of the interactive system from SH and ImH^+ is probably 3.1 and that its decomposition to S^- and Im is 9.6, i.e. a separation of 6.5 pK_a units. The wider separation of the two pK_a values in actinidin implies greater stabilization in the interactive system of this enzyme than obtains in either papain or ficin, and this greater stabilization might be expected to result in lower nucleophilicity of the active-centre sulphur atom in the interactive state.

As is the case for ficin (Malthouse & Brocklehurst, 1976; Brocklehurst & Malthouse, 1980), there is no group in actinidin with pK_a about 4 whose protonation greatly diminishes the reactivity of the active centre towards 2-pyridyl disulphide reactivity probes. Papain remains unique in this respect (Shipton & Brocklehurst, 1978; Brocklehurst & Malthouse, 1980).

As was found for reactions of both papain and ficin with 2-pyridyl disulphide probes, there is a tendency for data points in the pH range 4.5–5.5 to lie above the theoretical line in profiles like that in Fig. 1. Some possible causes of this phenomenon were discussed by Shipton & Brocklehurst (1978).

(*ii*) *Reactivities towards Nbf-Cl.* It seems probable that the additional group of pK_a 4 in papain whose protonation effectively switches off the reactivity of the interactive system towards the protonated 2-pyridyl disulphide reactivity probes might be the same group whose protonation results in the increase in the rate of the reaction of the interactive system with Nbf-Cl (Shipton *et al.*, 1976). The general acid-catalysed reaction of Nbf-Cl found with papain does not occur with ficin (Shipton *et al.*, 1976) or with actinidin. Typical second-order rate constants (k, in $M^{-1} \cdot s^{-1}$) for the three reactions of Nbf-Cl at 25.0°C, I 0.1 M in 6.7% (v/v) ethanol, are: for papain, pH 4.4, $k = 8.0$; pH 3.8, $k = 21.0$; pH 3.3, $k = 31.0$; for ficin, pH 5.1, $k = 0.3$; pH 4.0, $k = 0.23$; pH 3.3, $k = 0.09$ (see Shipton *et al.*, 1976); for actinidin, pH 5.73, $k = 0.6$; pH 4.38, $k = 0.15$. All reactions involved mainly thiol-group labelling ($\lambda_{max.}$ 420 nm for actinidin and ficin, as is the case for low-molecular-weight thiols, and the abnormally low value of $\lambda_{max.}$ 405 nm for papain). Amino-group labelling ($\lambda_{max.}$ 470 nm) becomes apparent gradually and only at pH values above 5. The difference in absorption spectra between *S*-Nbf-papain and the other two *S*-Nbf-enzymes points to a perturbing influence in the papain active centre, not present in

Table 2. *Second-order rate constants (k) for the reactions of the active-centre thiol groups of actinidin, ficin and papain with disulphide reactivity probes containing the 2-pyridyl moiety in 13.4% acetonitrile at 25°C at I 0.1*

Enzyme	Probe reagent	pH	$10^{-3}k$ ($M^{-1}\cdot s^{-1}$)	Reference
Actinidin	Py-S-S-Pr (Compound I)*	4.87	0.143	The present work
		5.06	0.061	
		5.20	0.044	
		6.74	0.033	
		5.71	0.031	
		6.07	0.030	
		6.90	0.034	
		7.04	0.028	
		7.15	0.023	
		7.55	0.042	
		7.67	0.059	
		8.65	0.165	
		8.86	0.240	
Actinidin	Compound (II)	5.00	2.20	
		5.15	1.96	
		5.76	1.25	
		6.04	0.89	
		6.90	0.69	
		7.08	0.38	
		7.90	0.46	
		8.16	0.47	
		8.93	0.99	
		9.95	1.25	
Ficin	Compound (II)	3.31	27.5	Brocklehurst & Malthouse (1980)
		3.95	17.8	
		5.61	14.8	
		6.52	13.7	
		7.79	10.0	
		9.86	26.0	
Papain	Compound (II)	3.20	13.0	Brocklehurst *et al.* (1979)
		3.81	26.8	
		4.28	41.7	
		6.00	52.0	
		8.30	33.0	
		8.93	14.5	

* Because it was necessary to the study the reactions of compound (II) in 13.4% (v/v) acetonitrile for solubility reasons, the effect of this solvent on the kinetics of the reaction of the thiol group of actinidin with Py-S-S-Pr was studied. As was found for the reaction of ficin with Py-S-S-Pr (Brocklehurst & Malthouse, 1980) and for the reaction of papain with 2,2′-dipyridyl disulphide (Brocklehurst *et al.*, 1979), the rate constants are smaller in 13.4% acetonitrile than in water, but the profile shape appears to be preserved (see Fig. 1).

the other two enzymes, at least in the conformations of these enzymes that predominate at the pH values where the reactions were studied.

(*iii*) *Reactivities towards compound (II).* Change in the structure of the reactivity probe from Py-S-S-Pr (compound I) to compound (II) results in a considerable change in the reactivity characteristics displayed towards the probe molecule by papain (Brocklehurst *et al.*, 1979). Thus, whereas the reaction of papain with Py-S-S-Pr is characterized by a rate minimum at pH 6, the reaction of papain with compound (II) is characterized by a striking rate maximum, the values of k at pH 6.15, I 0.1 M, 25°C, in 13.4% (v/v) acetonitrile being $k_{\text{Py-S-S-Pr}} = 1611\,M^{-1}\cdot s^{-1}$ (see Table 2) and $k_{\text{compound (II)}} = 50000\,M^{-1}\cdot s^{-1}$ (Brocklehurst *et al.*, 1979). This dramatic change in profile shape caused by the introduction of the hydrophobic side chain into the probe reagent is not observed in the corresponding reactions of either ficin (Brocklehurst & Malthouse, 1980) or actinidin (see Table 2). The feature of the papain active centre that provides for the high rate of the interactive system of papain with compound (II) is clearly absent in both ficin and actinidin.

(*iv*) *Possible differences in structure and mechanism.* Clearly the differences in character and reactivity between the interactive systems of

actinidin, ficin and papain must derive from differences in active-centre structure. The questions now posed, however, are to what extent are these differences mechanistically significant, and do these differences in reactivity characteristics add anything to current thinking about the two-state (UP–DOWN) mechanism for papain described by Angelides & Fink (1979)?

The two-state mechanism is attractive on theoretical grounds, particularly in the context of stereoelectronic considerations (see Brocklehurst *et al.*, 1981 and references therein) and could explain the lack of catalytic competence of thiosubtilisin (Brocklehurst & Malthouse, 1981).

It was pointed out by Angelides & Fink (1978) that the crystals of papain used for crystallographic study were grown at high pH (9.3), where the UP conformation, postulated by these authors to be catalytically inactive, should predominate. It is of considerable interest, therefore, that the crystals of actinidin used for crystallographic study (Baker, 1980) were grown at pH6 (Baker, 1973, 1976), where, if a conformation analogous to the postulated DOWN conformation of papain exists, it might be expected to predominate. In fact, the structure of actinidin at 0.17nm resolution reported by Baker (1980) is closely similar to the UP conformation of papain and does not provide any structural evidence for a DOWN conformation analogous to that proposed by Angelides & Fink (1978). The carboxy group of aspartic acid-161 (supposedly analogous to aspartic acid-158 in papain) is turned away from the active centre and hydrogen-bonds with the main-chain amido group of residue 139 (161-$O_{\delta 1}$''''139-N, 0.293nm). It is approx. 0.56nm from $N_{\delta 1}$ of histidine-162 and approx. 0.71nm from S_γ of cysteine-25. The main structural difference between the active centres of papain and actinidin deduced from crystallographic study lies not in the catalytic site, but beyond, in the hydrophobic binding site [the 'S_2 subsite' in the terminology of Berger & Schechter (1970)]. In papain, the S_2 subsite consists of a deep pocket lined with hydrophobic side chains of tyrosine-67, proline-68, tryptophan-69, phenylalanine-207, valine-133 and valine-157. Crystallographic study (Drenth *et al.*, 1976) of the binding of 'chloromethyl ketone' inhibitors show that the aromatic substituent of the inhibitor, and thus presumably of an analogous substrate, occupies this pocket, in van der Waals contact (0.35–0.45nm) with valine-133 and valine-157 and 0.45–0.65nm from tyrosine-67, proline-68 and tryptophan-69. In actinidin, the S_2 subsite is lined by the side chains of tyrosine-69, isoleucine-70, alanine-136, valine-160, alanine-163 and methionine-211. The major difference is that serine-205, the residue at the end of the S_2 subsite in papain, becomes methionine-211 in actinidin. This side chain stretches across the end of the pocket, making it noticeably shorter than in papain. Thus, as Baker *et al.* (1980) have pointed out, if substrates bind to actinidin in the same location as they appear to do in papain, an aromatic *N*-acyl substituent would approach to within 0.25nm of the side chain of methionine-211. Thus, in actinidin, either the methionine side chain must be displaced in the adsorptive complex or the binding modes in papain and actinidin must be different.

The low values of $k_{cat.}/K_m$ for the actinidin-catalysed hydrolysis of hydrophobic substrates relative to those for analogous papain-catalysed hydrolysis can be explained in terms of the difference in the binding sites of the two enzymes, and a similar explanation could be invoked to explain the lack of high rates of reaction of the actinidin thiol group towards compound (II) in neutral media. If this is the explanation for the lack of high reactivity towards compound (II), the similar lack of high reactivity in ficin (Brocklehurst & Malthouse, 1980) could point to a difference also between the S_2 subsites of papain and ficin. The results obtained with Py-S-S-Pr and Nbf-Cl, however, point to a difference between the catalytic sites of papain and actinidin, a difference involving interaction in the catalytic diad (Cys–His) or catalytic triad (Cys–His–Asp). The kinetic data obtained by using the two-protonic-state Py-S-S-Pr probe (for actinidin, the relatively low reactivity in the XH state and the large separation, 6.5 units, of the active-centre pK_a values) suggest greater stabilization and lower reactivity of the thiolate ion of the interactive system of actinidin than obtains in the interactive system of papain. If a DOWN conformation does exist in papain, the stabilizing effect on the thiolate anion of the ImH^+ cation might be modulated by the further association of that cation with the aspartate carboxylate anion. This could result in enhanced development of nucleophilic character in the 'less solvated' thiolate anion of the papain interactive system. The positively co-operative protonations in the pH region around 4 that characterize the reactions of the papain active centre with Py-S-S-Pr and with Nbf-Cl are not found with either actinidin or with ficin. These co-operative processes found in the papain reactions are nicely explained by the two-state (UP–DOWN) model for the papain active centre (though their existence does not, of course, prove the model), and the lack of evidence of the co-operative processes in the corresponding reactions of actinidin and ficin points to a significant difference in active-centre structure. The structural work on actinidin suggests the simplest explanation of these differences in behaviour, which is that actinidin exists even in neutral and acidic media in an UP conformation, and the basis proposed for the co-operativity in papain does not exist in actinidin. If an UP–DOWN mechanism does exist for ficin (for

which no crystallographic data are available), its aspartate carboxy group probably has a low pK_a value (less than 3; Brocklehurst & Malthouse, 1980). If evidence for an UP–DOWN mechanism for actinidin is eventually forthcoming, e.g. from cryokinetic experiments, a similar conclusion of a low pK_a for the aspartate carboxy group would be required, but it would be necessary also to propose that actinidin is stabilized in the crystal in a conformation that is very different from the catalytically active conformation that might predominate in solution. If this were found to be the case, it would have rather serious consequences for the central relevance of crystallographic studies to the study of enzyme mechanism.

The present state of the accumulated kinetic and structural evidence is that actinidin seems to contain a nucleophilic interactive cysteine–histidine system in an UP conformation, and the papain active centre seems to be uniquely different in ways that could be explained by an UP–DOWN mechanism.

These findings, that the active centres of some cysteine proteinases behave differently towards certain reactivity probes in the ways described, suggests the interesting possibility that there may be (at least) two ways in which acylation of a sulphur nucleophile might be achieved in these enzymes. One way, that which has been proposed for papain, involves active-centre isomerization to provide synchronous or preprotonation of the leaving group in accord with the predictions of stereoelectronic theory. Another way, which would not require this type of isomerization, might involve a strain mechanism like that proposed for the serine proteinases (Kraut, 1977) in which the substrate is rendered highly electrophilic. The necessity for synchronous protonation of the leaving group might then be obviated. In this event, however, the problem of the lack of catalytic ability of thiolsubtilisin despite its possession of a highly nucleophilic interactive system (Brocklehurst & Malthouse, 1981) would still remain unresolved.

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