The interplay of electrostatic fields and binding interactions determining catalytic-site reactivity in actinidin

A possible origin of differences in the behaviour of actinidin and papain

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1. The pH-dependence of the second-order rate constant (k) for the reaction of actinidin (EC 3.4.22.14) with 2-(*N'*-acetyl-L-phenylalanyl-amino)ethyl 2'-pyridyl disulphide was determined and the contributions to k of various hydronic states were evaluated. 2. The data were used to assess the consequences for transition-state geometry of providing P_2/S_2 hydrophobic contacts in addition to hydrogen-bonding opportunities in the S_1-S_2 intersubsite region. 3. The P_2/S_2 contacts (a) substantially improve enzyme–ligand binding, (b) greatly enhance the contribution to reactivity of the hydronic state bounded by pK_a 3 (the pK_a characteristic of the formation of catalytic-site $-S^-/-ImH^+$ state) and pK_a 5 (a relatively minor contributor in reactions that lack the P_2/S_2 contacts), such that the major rate optimum occurs at pH 4 instead of at pH 2.8–2.9, and (c) reveal the kinetic influence of a pK_a approx. 6.3 not hitherto observed in reactions of actinidin. 4. Possibilities for the interplay of electrostatic effects and binding interactions in both actinidin and papain (EC 3.4.22.2) are discussed.

INTRODUCTION

The structural variation that exists within the cysteine proteinase family provides a range of possibilities for investigation of the consequences of different electrostatic effects and of different binding opportunities for active-centre chemistry and catalytic mechanism [see Brocklehurst (1986, 1987) and Brocklehurst *et al.* (1987b) for reviews].

The two closely similar enzymes papain (EC 3.4.22.2) and actinidin (EC 3.4.22.14) are of particular interest because, although their crystal structures are closely similar and virtually superimposable in the active-centre regions (see Kamphuis *et al.*, 1985; Baker & Drenth, 1987), evidence for substantial differences in the characteristics and behaviour of their catalytic sites continues to accumulate (Salih *et al.*, 1987; Brocklehurst *et al.*, 1988b). This includes observation of marked differences in (a) the electrical properties of the two catalytic-site microenvironments as assessed by approximately isosteric neutral and anionic reactivity probes (Brocklehurst *et al.*, 1983), (b) the responses of the two enzymes to two hydronic dissociations, one with pK_a approx. 4 and one with pK_a 5.0–5.5 as assessed by a thiol-located spectroscopic reporter group (Brocklehurst *et al.*, 1984), pH-dependent reactivity characteristics towards different types of reactivity probe (Salih & Brocklehurst, 1983; Salih *et al.*, 1987; Brocklehurst *et al.*, 1988b), and pH-dependence of k_{cat}/K_m (Salih *et al.*, 1987), and (c) the susceptibility of reactions of the two enzymes to change in transition-state geometry, and to a switch of a dominant kinetically influential pK_a , consequent upon pro-

vision of opportunities for hydrogen-bonding interactions across the interdomain cleft in the S_1-S_2 (Berger & Schechter, 1970) intersubsite region (Brocklehurst *et al.*, 1988a,b).

The importance of the amide bond adjacent to the scissile bond of a substrate as a sensitive mechanism through which the effect of hydrophobic residues at P_2 may be relayed to the catalytic site was established for papain by Lowe & Yuthavong (1971), and the ways in which their steady-state kinetic data identified the problems that seem amenable to study by substrate-derived 2-pyridyl disulphide reactivity probes was discussed by Brocklehurst *et al.* (1988a).

In the present paper we report the results of a pH-dependent kinetic study of the reaction of actinidin with 2-(*N'*-acetyl-L-phenylalanyl-amino)ethyl 2'-pyridyl disulphide (compound I) and hence the consequences for transition-state geometry in some reactions of the actinidin catalytic site of providing for P_2/S_2 hydrophobic contacts in addition to hydrogen-bonding opportunities in the S_1-S_2 intersubsite region. The P_2/S_2 contacts (a) substantially improve enzyme–ligand binding, (b) greatly enhance the contribution to reactivity of the hydronic state bounded by pK_a 3 (the pK_a characteristic of the formation of catalytic site $-S^-/-ImH^+$ state) and pK_a 5 (a relatively minor contributor in reactions that lack the P_2/S_2 contacts), such that the major rate optimum occurs at pH 4 instead of at pH 2.8–2.9, and (c) reveal the kinetic influence of a pK_a approx. 6.3 not hitherto observed in reactions of actinidin. Possibilities for the interplay of electrostatic effects and binding interactions in both actinidin and papain are discussed.

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MATERIALS AND METHODS

The purification of actinidin, the synthesis and characterization of compound (I) and details of the kinetic methods have been reported previously (Brocklehurst *et al.*, 1988b, and references cited therein). The reaction of actinidin with compound (I) was studied at 25 °C at 1.0 l under pseudo-first-order conditions with [actinidin] = 3–5 μ M and [compound (I)] = 30–115 μ M. Linear dependence of $k_{\text{obs.}}$ (s^{-1}) on [compound (I)] was demonstrated at pH values 4.0 and 6.0. The pH-dependence of the second-order rate constant (k) for this reaction was evaluated by using the eight-parameter (four- pK_a) rate equation (eqn. 1) discussed by Brocklehurst & Brocklehurst (1988) and a BBC Microcomputer (model B) fitted with a 6502 second processor. The computer program provides a display of the contributions to k of the terms of eqn. (1) associated with each of the pH-independent rate constants, $\tilde{k}_{\text{XH}_3}$ – $\tilde{k}_{\text{X}}$, each characteristic of a particular hydronic state, in addition to the complete pH– k profile:

$$k = \frac{\tilde{k}_{\text{XH}_3}}{1 + \frac{[\text{H}^+]}{K_{\text{XH}_3}} + \frac{K_{\text{XH}_3}K_{\text{XH}_2}}{[\text{H}^+]^2} + \frac{K_{\text{XH}_3}K_{\text{XH}_2}K_{\text{XH}}}{[\text{H}^+]^3}} + \frac{\tilde{k}_{\text{XH}_2}}{1 + \frac{[\text{H}^+]^2}{K_{\text{XH}_4}K_{\text{XH}_3}} + \frac{[\text{H}^+]}{K_{\text{XH}_3}} + \frac{K_{\text{XH}_2}}{[\text{H}^+]} + \frac{K_{\text{XH}_2}K_{\text{XH}}}{[\text{H}^+]^2}} + \frac{\tilde{k}_{\text{XH}}}{1 + \frac{[\text{H}^+]^3}{K_{\text{XH}_4}K_{\text{XH}_3}K_{\text{XH}_2}} + \frac{[\text{H}^+]^2}{K_{\text{XH}_3}K_{\text{XH}_2}} + \frac{[\text{H}^+]}{K_{\text{XH}_2}} + \frac{K_{\text{XH}}}{[\text{H}^+]}} + \frac{\tilde{k}_{\text{X}}}{1 + \frac{[\text{H}^+]^4}{K_{\text{XH}_4}K_{\text{XH}_3}K_{\text{XH}_2}K_{\text{XH}}} + \frac{[\text{H}^+]^3}{K_{\text{XH}_3}K_{\text{XH}_2}K_{\text{XH}}} + \frac{[\text{H}^+]^2}{K_{\text{XH}_2}K_{\text{XH}}} + \frac{[\text{H}^+]}{K_{\text{XH}}}} \quad (1)$$

RESULTS AND DISCUSSION

Consequences for the pH-dependence of actinidin reactivity of augmentation of trans-cleft hydrogen-bonding interactions with hydrophobic P_2/S_2 contacts

These consequences were evaluated by studying the pH-dependence of k for the reaction of the catalytic-site thiol group of actinidin with compound (I) and comparing the shape of the pH– k profile (Fig. 1b) and the characterizing parameters of its constituent hydronic states (Tables 1 and 2) with those of the pH– k profiles for the analogous reactions (Brocklehurst *et al.*, 1988b) with compounds (II) and (III) (Fig. 2).

Fig. 1(a) shows a fit to the data for the reaction of actinidin with compound (I) that involves three of the four pK_a values (approx. 2.5, 3 and 5) found to be characteristic of the reactions of actinidin with a number of alkyl 2-pyridyl disulphides (Brocklehurst *et al.*, 1988b). For these other reactions a pK_a value of 9.5 clearly characterizes an increase in k as the pH is increased in alkaline media. Convincing evidence for this pK_a does

not exist in Fig. 1, although a small sigmoid wave between pH 9 and pH 10 may be a possibility. Presumably, the apparent absence of pK_a 9.5 means that the reactivity of the most hydron-deficient state (the X state, containing the uncomplicated thiolate anion of actinidin) is not very different from a presumed hydronic state containing the $-\text{S}^-/\text{ImH}^+$ ion-pair of actinidin, whose dehydration is characterized by pK_a 9.5 as evidenced from others reactions of this enzyme.

The other striking feature of Fig. 1(a) is the failure of the data to fit the line corresponding to eqn. (1) in the pH range 5.5–8 when only pK_a values 2.49, 3.0 and 5.1 are used. Fig. 1(b) shows that a good fit to the data over the whole pH range requires an additional pK_a with a value approx. 6.3.

Fig. 2 shows the changes in the shape of the pH– k profile for three reactions of actinidin from that (Fig. 2, curve a) with a 'featureless' probe [compound (III)] containing a simple hydrocarbon (n-propyl) side chain to

that (Fig. 2, curve b) with a probe containing a $\text{P}_1\text{--P}_2$ amide bond [compound (II)], and further to that (Fig. 2, curve c) with a probe containing both a $\text{P}_1\text{--P}_2$ amide bond and an L-phenylalanine side chain as a potential occupant for the hydrophobic pocket of the S_2 subsite (see Scheme 1). The ordinate axis has the same scaling factor for curves a and b in Fig. 2 ($10^{-3}k$) so that values of k are directly comparable. The analogous scaling factor used for curve c in Fig. 2 is $2 \times 10^{-4}k$. This permits all three profile shapes to be compared easily, but it should be noted that this reaction is considerably faster than the other two.

The computer program used in connection with eqn. (1) (Brocklehurst & Brocklehurst, 1988) permits the contributions of individual hydronic states to be displayed, and Fig. 1(b) shows the dominance of the hydronic state bounded by pK_a values 3.1 and 5.0. One interesting feature of Fig. 2 is that this same hydronic state is sufficiently developed in the reaction of compound (II) (Fig. 2, curve b) that a sigmoid wave in this profile is clearly discerned in the pH region approx. 3.5–5 above the profile for the reaction of compound (III) (Fig. 2, curve a). The data in Figs. 1 and 2 suggest that binding of a $\text{P}_1\text{--P}_2$ amide bond in the interdomain cleft of actinidin enhances the effectiveness of the hydronic state bounded by pK_a values 3 and 5, and that the additional binding of a hydrophobic side chain in the S_2 subsite increases the effectiveness of this hydronic state so much that it becomes the dominant hydronic state in the reaction with compound (I). Accordingly, the rate maximum for the reaction of actinidin with compound (I) occurs at pH 4, whereas for the reactions with compounds (II) and (III) it occurs at pH 2.8–2.9. Another striking characteristic of the reaction of actinidin with compound (I), which has no analogue in the reactions with com-

Fig. 1. pH-dependence of the second-order rate constant (k) for the reaction of actinidin with 2-(*N'*-acetyl-L-phenylalanyl-amino)ethyl 2'-pyridyl disulphide (compound I) at 25 °C and 0.1 M in aqueous buffers

The points are experimental; the three points at pH values approx. 3.1–3.5 that lie substantially above the rest were collected as part of a supplementary data set, the rest of which coalesced with the initial data set as shown. The continuous lines are theoretical for eqn. (1) of the text (b) or for a truncated form appropriate for a three- pK_a model (a) and the following values of the parameters (with $\tilde{k}$ values in $M^{-1}\cdot s^{-1}$): for (a) $\tilde{k}_{XH_2} = 1.2 \times 10^5$, $\tilde{k}_{XH} = 3.6 \times 10^5$, $\tilde{k}_X = 5.0 \times 10^4$, $pK_{XH_2} = 2.49$, $pK_{XH} = 3.1$ and $pK_{XH} = 5.0$, and for (b) $\tilde{k}_{XH_2} = 1.2 \times 10^5$, $\tilde{k}_{XH_2} = 3.6 \times 10^5$, $\tilde{k}_{XH} = 1.3 \times 10^5$, $\tilde{k}_X = 5.0 \times 10^4$, $pK_{XH_2} = 2.49$, $pK_{XH_2} = 3.1$, $pK_{XH_2} = 5.0$ and $pK_{XH} = 6.3$. The possibility of an additional pK_a approx. 9.5 (see the text) is not depicted. The broken lines (i)–(iv) in (b) correspond to the contributions to k of the individual hydronic states of the reaction that are provided by the terms in eqn. (1) associated with $\tilde{k}_{XH_2}$ – $\tilde{k}_X$ respectively.

pounds (II) and (III), is the substantial contribution to k of a hydronic state bounded by pK_a values 5.0 and 6.3 that produces marked asymmetry in the bell-shaped component of the pH- k profile in the pH region 5–6 (Figs. 1a and 1b). The other remarkable feature of Fig. 1 (and Fig. 2, curve c) is the fact that k is essentially pH-independent in the pH range 7.5–10, which results in the apparent absence of the expected kinetically influential pK_a of 9.5.

Fig. 2. Variation in the shape of the pH- k profile for reactions of actinidin with change in probe structure (R-S-S-2-Py)

The continuous lines correspond to eqn. (1) of the text and the following values of the characterizing parameters (with $\tilde{k}$ values in $M^{-1}\cdot s^{-1}$): for curve a, where $R = CH_3-CH_2-CH_2$ (compound III), $\tilde{k}_{XH_2} = 8.0 \times 10^3$, $\tilde{k}_{XH_2} = 7.0 \times 10^2$, $\tilde{k}_{XH} = 8.0 \times 10^2$, $\tilde{k}_X = 1.5 \times 10^3$, $pK_{XH_2} = 2.73$, $pK_{XH_2} = 3.1$, $pK_{XH_2} = 5.2$ and $pK_{XH} = 9.5$; for curve b, where $R = CH_3-CO-NH-CH_2-CH_2$ (compound II), $\tilde{k}_{XH_2} = 6.0 \times 10^3$, $\tilde{k}_{XH_2} = 1.25 \times 10^3$, $\tilde{k}_{XH} = 4.2 \times 10^2$, $\tilde{k}_X = 3.4 \times 10^3$, $pK_{XH_2} = 2.48$, $pK_{XH_2} = 3.15$, $pK_{XH_2} = 5.2$ and $pK_{XH} = 9.5$ (Brocklehurst *et al.*, 1988b); for curve c, where $R = CH_3-CO-NH-CH(CH_2Ph)-CO-NH-CH_2-CH_2$ (compound I), the values are those given in Fig. 1(b) legend. The arrows define the limits of the data.

The structural features of the non-pyridyl part of compound (I) appear to engage in binding interactions with actinidin that produce progressively rate enhancements in those hydronic states bounded by (a) pK_a 6.3 and 9.5, (b) pK_a 5.0 and 6.3, and (c) pK_a 3.1 and 5.0.

More detailed comparison of reactivities is assisted by consideration of Tables 1 and 2. The kinetically influential pK_a values of 5 and 6.3 are depicted in Table 2 as being associated with the ionization of two carboxy groups ($-\overset{\alpha}{CO_2H}$ and $-\overset{\beta}{CO_2H}$). This is discussed below, after the kinetic effects have been considered, in connection with known structural differences between actinidin and papain. The second-order rate constant characteristic of an individual hydronic state ($\tilde{k}$) is usefully considered as a ratio of the first-order rate constant (k_{+2}) for reaction within an assumed adsorptive complex and the dissociation constant of this complex ($K_r = k_{-1}/k_{+1}$) (see Brocklehurst *et al.*, 1987a). [k_{+2} and K_r relate to the simplest intermediate complex model for a time-dependent modification reaction of an enzyme (E) by a site-specific reagent (R), i.e. $E + R \xrightleftharpoons{K_r} ER \xrightarrow{k_{+2}} \text{products}$.]

Variation of values of $\tilde{k}_X$ with change in probe structure are accounted for most simply mainly in terms of a decrease in K_r with increase in binding opportunities, although effects on k_{+2} may also need to be considered. For actinidin (Tables 1 and 2) replacement of the n-propyl side chain in compound (III), firstly with the side chain in compound (II) that provides opportunities for trans-cleft hydrogen bonding and then with the side chain in compound (I) that provides in addition for the possibility of hydrophobic P_2/S_2 contacts, results in increases in $\tilde{k}_X$ by factors of 2-fold and 35-fold re-

Scheme 1. Schematic drawing showing the S₂ subsite, S₁-S₂ intersubsite and catalytic-site regions of actinidin and papain with 2-(N-acetyl-L-phenylalanyl-amino)ethyl 2'-pyridyl disulphide (compound I) bound so as to provide an -ImH⁺-assisted transition state

The residues of the S₂ subsite that are expected to make most intimate contacts with a non-polar side chain in the P₂ position are underlined. In both enzymes these are all hydrophobic, which provides a common non-polar binding pocket. The most marked difference is that in actinidin the thioether side chain of Met-211, the residue that corresponds to Ser-205 in papain, lies across the back of the binding pocket, making it significantly shorter. This Scheme is based on a schematic drawing of a papain-substrate complex that was kindly supplied by Professor Jan Drenth and Dr. Ted Baker.

Table 1. Effects of providing binding opportunities on the characteristics of the reactions of the catalytic-site thiol group of actinidin with 2-pyridyl disulphides (R-S-S-2-Py) at 25 °C and /0.1

The various hydronic states are designated X-XH₄ to indicate their relative stoichiometries in hydrons; the XH₄ state is unreactive.

Reactivity probe	Macroscopic pK _a values				pH-independent rate constants (M ⁻¹ ·s ⁻¹)			
	pK _{XH₄}	pK _{XH₃}	pK _{XH₂}	pK _{XH}	10 ⁻³ $\tilde{k}_{XH_3}$	10 ⁻³ $\tilde{k}_{XH_2}$	10 ⁻³ $\tilde{k}_{XH}$	10 ⁻³ $\tilde{k}_X$
(III) CH ₃ -CH ₂ -CH ₂ -S-S-2-Py	2.73*	3.1	5.2	9.5	8.0	0.7	0.8	1.5
(II) CH ₃ -C(=O)-NH-CH ₂ -CH ₂ -S-S-2-Py	2.48*	3.15	5.2	9.5	6.0	1.25	0.42	3.4
(I) CH ₃ -C(=O)-NH-CH(CH ₂ Ph)-C(=O)-NH-CH ₂ -CH ₂ -S-S-2-Py	2.49*	3.1	5.0	6.3	140	330	150†	52

* These pK_a values characterize the conjugate acids of the various probes hydronated on the pyridyl N atom (see Brocklehurst *et al.*, 1988b).

† This value of $\tilde{k}_{XH}$ characterizes the hydronic states bounded by pK_a values 5.0 and 6.3, whereas the other two values of $\tilde{k}_{XH}$ characterize hydronic states bounded by pK_a values 5.2 and 9.5.

spectively. These increases are considerably smaller than those found for the corresponding reactions of papain (factors of 11-fold and 333-fold respectively; Brocklehurst *et al.*, 1988b). In the X state the reaction is considered to proceed through a transition state in which the catalytic-site thiolate anion attacks the S atom of the probe distal from the pyridyl ring with some charge development in and around the pyridyl ring of the 2-mercaptopyridine leaving group. The values of $\tilde{k}_X$ for the reactions of both actinidin and papain towards

the featureless probe (compound III) are similar [1.5×10^3 M⁻¹·s⁻¹ for the actinidin reaction (Table 1), and 1.8×10^3 M⁻¹·s⁻¹ for the papain reaction (Brocklehurst *et al.*, 1988b)]. One factor that might be predicted to contribute to the considerably smaller values of $\tilde{k}_X$ for actinidin reactions than for papain reactions when specific binding opportunities are present in the probe [for compound (II) $\tilde{k}_X$ (actinidin) = 3.4×10^3 M⁻¹·s⁻¹ and $\tilde{k}_X$ (papain) = 1.95×10^4 M⁻¹·s⁻¹, and for compound (I) $\tilde{k}_X$ (actinidin) = 5.2×10^4 M⁻¹·s⁻¹ and $\tilde{k}_X$

Column a: the thiol group is undissociated in this hydronic state, which therefore is of negligible nucleophilicity. Column b: the ionic form depicted is the only one containing a thiolate anion and thus the only one in this hydronic state predicted to possess appreciable nucleophilic character; it is a 'minor' hydronic form resulting from 'crossed-over' pK_a values (see Malthouse & Brocklehurst, 1976) because pK_a 2.5–2.7 characterizes hydronic dissociation of R–S–S–2-PyH⁺. Column c: in this hydronic state formation of both $-\text{ImH}^+/-\text{S}^-$ and R–S–S–2-Py by the two dehydrations is predicted to be substantially complete; a minor ionic form is one in which R–S–S–2-PyH⁺ is retained and one of the postulated carboxy groups is ionized. Column d: a minor ionic form contains $-\text{CO}_2^-$ instead of $-\text{C}_2^-$; in the reaction of probe II, pK_a 6.3 is not kinetically influential and it is assumed that the reactivity of this hydronic state is similar to that in column e; in the reaction of probe I, no change in reactivity across pK_a 9.9.5 is observed and the reactivity in this hydronic state is taken as the value of k_x (Table 1). Column f: in this hydronic state (the X state) all four dissociating groups whose kinetic influence is detected by sigmoid waves in the pH– k profiles are hydron-deficient. [I] denotes unity by definition.

$$(I) \text{ (R = CH}_3\text{--}\overset{\text{=}}{\text{C}}\text{--NH--CH--}\overset{\text{=}}{\text{C}}\text{--NH--CH}_2\text{--CH}_2\text{)} \\ \text{CH}_2\text{Ph}$$

(papain) = $6 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$] would be the presence in particular locations of additional negative charges in actinidin. If the binding interactions promote changes in transition-state geometry (k_{+2} effects) in addition to providing an increase in the steady-state (or quasi-equilibrium) concentration of adsorptive complex (K_r effect), one or more of the uniquely located negative charges in actinidin might be positioned such that the rearranged anionic transition state is destabilized. Thus, even if the K_r effects are comparable for both enzymes, adverse k_{+2} effects in actinidin would provide a decreased sensitivity to increase in k consequent upon providing binding opportunities in the probe molecule. Structural evidence for the presence of differently located anionic groups in actinidin is discussed below.

In the other reactive hydronic states of these reactions (XH_n where $n > 0$) k_{+2} effects might be expected to be even more influential because of the possibility of transition-state stabilization when binding effects provide for contact of the pyridyl N atom of the leaving group with the $-\text{ImH}^+$ side chain of the catalytic site. Evidence for this type of transition-state stabilization in reactions of papain with substrate-derived 2-pyridyl disulphides is particularly convincing because introduction of the structural features present in compounds (I) and (II) results in the production of striking rate maxima in pH- k profiles at pH 6-7, which contrast with the rate minima in reactions of 'featureless' probes such as compound (III) (see Brocklehurst *et al.*, 1988b). These rate maxima occur in hydronic states containing $-\text{S}^-/-\text{ImH}^+$ ion-pairs and non-hydrated probe molecules, which provide for the type of transition-state stabilization indicated above.

Differential changes in the reactivities of the various hydronic states of reaction consequent upon structural change in the probe molecule (as in Fig. 2, curve a, and Table 1) are indicative of the existence of k_{+2} effects in addition to the reasonable assumption of K_r effects. In view of the analysis of the corresponding reactions of papain referred to above (Brocklehurst *et al.*, 1988b), it seems appropriate to suggest that the rate enhancements observed in several hydronic states of the reaction of actinidin with compound (I) (see Fig. 1b and Table 2) might arise from contributions to k in particular pH regions from $-\text{ImH}^+$ -assisted transition states, modulated by the ionization of two groups associated with macroscopic pK_a values of 5 and 6.3 (plausibly carboxy groups in perturbing environments). Thus the complex pH- k profile of Fig. 1(b) could be interpreted as follows. The reaction of $-\text{S}^-/-\text{ImH}^+$ with the hydronated probe molecule [component (i), a 'minor' hydronic state with 'crossed-over' pK_a values] is augmented substantially by reaction with the non-hydrated probe in a major hydronic state as the concentration of $-\text{S}^-/-\text{ImH}^+$ ion-pair increases across pK_a 3. This part of the pH- k profile may be analogous to the acid limb of the profile for the corresponding reaction of papain (Fig. 3), where, however, the pK_a of $-\text{SH}/-\text{ImH}^+$ is about 1 unit higher. In the case of actinidin both postulated carboxy groups (α and β in Table 2) would be largely un-ionized in the pH range covered by the acid limb of the profile. The most marked difference in shape between the profiles for actinidin and papain (Fig. 3) occurs at $\text{pH} > 4$, where k decreases markedly in the actinidin profile across pK_a 5 as one of postulated carboxy groups becomes ionized [component (ii) in Fig. 1(b)]. One possibility is that the change in transition-state geometry promoted by the

Fig. 3. Comparison of the shapes of the pH- k profiles for the reactions of 2-(*N'*-acetyl-L-phenylalanyl-amino)ethyl 2'-pyridyl disulphide (compound I) with (curve a) actinidin and (curve b) papain

The lines correspond to eqn. (1) of the text and the following values of the characterizing parameters (with $\bar{k}$ values in $\text{M}^{-1} \cdot \text{s}^{-1}$): for curve a, the values given in Fig. 1(b) legend; for curve b, $\bar{k}_{\text{XH}_3} = 3.0 \times 10^6$, $\bar{k}_{\text{XH}_2} = 6.1 \times 10^6$, $\bar{k}_{\text{XH}} = 2.3 \times 10^6$, $\bar{k}_{\text{X}} = 6.0 \times 10^5$, $\text{pK}_{\text{XH}_4} = 3.9$, $\text{pK}_{\text{XH}_3} = 4.1$, $\text{pK}_{\text{XH}_2} = 8.25$ and $\text{pK}_{\text{XH}} = 9.5$ (Brocklehurst *et al.*, 1988b). The arrows define the limits of the data.

binding interactions in and around the S_2 subsite is inhibited by the new contribution to the electrostatic field to which the catalytic site is exposed, consequent upon ionization of one of the postulated carboxy groups in actinidin. A simple mechanism for this effect would be a repositioning of $-\text{ImH}^+$, caused by the influence of $-\text{CO}_2^-$, such that hydrogen bonding with the pyridyl N atom does not occur or is less effective. Ionization of the second carboxy group (formally associated with pK_a 6.3) appears to diminish the inhibitory effect of the first carboxylate anion (produced by the ionization of pK_a 5), resulting in additional asymmetry in Fig. 1(b) in the pH range 5-7 [see component (iii), $\bar{k}_{\text{XH}}$ in Table 1 and column 3 in Table 2]. This might be due to electrostatic repulsions and consequent conformational adjustments in the catalytic-site region. Thus the complexity of the pH- k profile in Fig. 1(b) could arise from the interplay of binding interactions and electrostatic effects in the field to which the catalytic site is exposed.

Towards a structural interpretation of the kinetic data

The kinetic data discussed above demonstrate the existence of kinetically influential ionizing groups in actinidin associated with pK_a values of 5 and 6.3. A pK_a value of 5-5.5 has been observed also in pH- k profiles of some reactions of papain (Brocklehurst *et al.*, 1988a), but only in sigmoid waves of small amplitude and only in a few reactions of papain. For actinidin, the kinetic effect of hydronic dissociation across pK_a 5 is much larger and occurs much more generally. These pK_a values suggest that they might characterize hydronic dissociation of carboxy groups in environments that either stabilize the undissociated carboxy group by accepting a hydrogen bond from it or destabilize the carboxylate anion, as would occur in a hydrophobic location or when two carboxylate anions are juxtaposed. It may be relevant in this connection that actinidin possesses many more

Table 3. Sequence-aligned residues in actinidin and papain that contain a carboxylic side chain in one or both of the enzymes with those containing a carboxylic side chain in only one of the two enzymes shown in capitals

The sequence alignment is that reported by Kamphuis *et al.* (1985).

Actinidin	Papain
Ser-3	GLU-3
Asp-6	Asp-6
ASP-15	Pro-15
GLU-21	Ser-21
Glu-35	Glu-35
Ser-47	*GLU-47
Glu-50	Glu-50
Glu-52	Glu-52
Asp-55	Asp-55
Gly-57	ASP-57
ASP-66	Asn-64
ASP-72	Ser-70
ASP-80	Tyr-78
GLU-86	Arg-83
GLU-87	Asn-84
ASP-95	Gln-92
ASP-97	Tyr-94
ASP-99	Arg-96
Leu-102	GLU-99
ASP-104	Gly-101
Asp-111	Asp-108
ASP-114	Arg-111
Glu-121	*Glu-118
Asp-138	*Glu-135
ASP-142	Lys-139
Ala-143	ASP-140
Asp-161	Asp-158
GLU-172	—
ASP-176	Gly-169
ASP-185	Gly-178
Glu-190	Glu-183
GLU-191	Asn-184

* Glutamic acid according to the nucleotide sequence reported by Cohen *et al.* (1986); residue 86 was reported as aspartic acid by Carne & Moore (1978) from the amino acid sequence and as glutamic acid (or possibly glutamine) by Baker (1980) from the X-ray-diffraction data.

carboxylic side chains (27) than does papain (16). The preponderance of anionic side chains over cationic side chains in actinidin provides for its low isoelectric point (3.1) (McDowall, 1970), which contrasts with the much higher value (8.75) for papain.

The amino acid residues of both actinidin and papain that contain carboxylic side chains (aspartic acid and glutamic acid) are shown together with the corresponding sequence-aligned residue in the other enzyme in Table 3. The three-dimensional structures of the two enzymes have been compared in detail by Baker (1981) and by Kamphuis *et al.* (1985) and further discussed by Baker & Drenth (1987) and by Brocklehurst *et al.* (1987b). Two details require comment to update previous discussions of the electrostatic fields on these enzymes and to focus attention on possible carboxy groups of interest. Firstly, the actinidin sequence shown by Kamphuis *et al.* (1985) and reproduced by Brocklehurst *et al.* (1987b) shows residue 86 to be glutamic acid. Residue 86 in the original

amino acid sequence given by Carne & Moore (1978) was reported as aspartic acid. Baker (1980), however, pointed out that the refinement of the actinidin structure at 0.17 nm resolution clearly shows the side chain of residue 86 to possess an additional carbon atom consistent with either glutamic acid or glutamine, and he assigned residue 86 as glutamic acid to preserve the same charge as in the chemical sequencing. In addition, he pointed out that if this is a carboxy group it is close enough to that of Glu-50 to enable them to share a hydron, with one of the pK_a values of this hydrogen-bonded system being abnormally high (perhaps approx. 6). Secondly, as pointed out by Brocklehurst *et al.* (1989) and discussed by Pickersgill *et al.* (1989), the nucleotide sequence of papain (Cohen *et al.*, 1986) has the codon for glutamic acid at amino acid positions 47, 118 and 135 whereas the amino acid sequence has glutamine at these three positions. These residues and residue 86 in actinidin are shown as glutamic acid in Table 3. Table 3 identifies residues with carboxylic side chains in actinidin that have no sequence-aligned analogue in papain. Such residues provide potential candidates for consideration as providing at least one of the hydronic dissociations with pK_a values 5 and 6.3 responsible for the unique kinetic behaviour of actinidin, although common sequence-aligned residues in different microenvironments must also be a possibility (see below).

The particular importance of the electrostatic fields to which the active-centre regions of actinidin and papain are exposed in determining their characteristics has been examined both by experiment (e.g. Brocklehurst *et al.*, 1983) and by calculation (e.g. Lavery *et al.*, 1983; Pickersgill *et al.*, 1988), and has been discussed by a number of authors [see Kamphuis *et al.* (1985), Baker & Drenth (1987) and Brocklehurst *et al.* (1987b) for reviews and Brocklehurst *et al.* (1989) and Pickersgill *et al.* (1989) for comment]. Examples of differences in residues near the active-centre clefts of actinidin and papain that might provide local differences in electrostatic properties, with the approximate distance from the thiol group of Cys-25 in parentheses, are: Ser-21 in papain corresponds to Glu-21 in actinidin (0.14 nm), and Asn-64 in papain corresponds to Asp-66 in actinidin (0.12 nm). Both of these differences are in the L-domain on the Cys-25 side of the cleft. In the R-domain, which contributes His-159, Lys-139 in papain corresponds to Asp-142 in actinidin. The amino acid sequence suggested that Asp-138 in actinidin (0.11 nm), which corresponds to Gln-135 in papain, is another electrostatic difference, but the nucleotide sequence of papain reports residue 135 as glutamic acid.

Examination of the structural data reveals several sets of residues that might account for the unique behaviour of actinidin as expressed through kinetic consequences of hydronic dissociation with pK_a values 5 and 6.3, where the dissociation across pK_a 6.3 appears to affect the consequence of hydronic dissociation across pK_a 5 (see above). An obvious feature to look for is one containing carboxy groups in close proximity, at least one of which is unique to actinidin, possibly in a hydrophobic environment. Three such sets of residues in actinidin are: (a) the surface pair Asp-80 (Tyr-78 in papain) and Asp-104, (b) Asp-55 (Asp-55 in papain) and Asp-95 (Gln-92 in papain), and (c) Glu-50 (Glu-50 in papain) and Glu-86 (Arg-83 in papain). Asp-80 and Asp-104 are surface residues and remote from the active centre, and are probably not strong candidates. Asp-55 and Asp-95 are close together in a hydrophobic environment but still

remote from the active centre. The remaining system (Glu-50 and Glu-86), which is part of a wider electrostatic net involving Lys-17 (Lys-17 in papain), Lys-181 (Lys-174 in papain) and Glu-35 (Glu-35 in papain), is particularly worthy of consideration. Baker (1980) had pointed out this electrostatic network as an interesting collection of buried charged groups within the actinidin structure, without any known functional role.

Glu-35, Glu-50 and Lys-181 all lie within the inter-domain interface along with Lys-17, partially buried at the end of the interface, with Glu-86 beyond. The charge on the two buried carboxylate side chains of Glu-35 and Glu-50 should be partly neutralized by Lys-17 and additionally by Lys-181, which may interact with Glu-35 through two water molecules, which may have a charge spreading role as discussed by Finney (1977). The other buried carboxy group (Glu-52) is in a partly hydrophobic environment adjacent to Ile-83, and Phe-76 and has no obvious cationic partner. The possibility that the Glu-50/Glu-86 pair might contribute an abnormally high pK_a was pointed out above.

Of the common sequence-aligned residues with ionizable side chains, the most obvious electrostatic influence on the catalytic-site $-S^-/-ImH^+$ ion-pairs of actinidin and papain has long been considered to be the neighbouring carboxylate anion (Asp-158 in papain and Asp-161 in actinidin) [see Brocklehurst *et al.* (1987b) for a review]. For papain it was suggested to be responsible for the second ionization with pK_a approx. 4, additional to that characteristic of $-SH/-ImH^+$, that influences both thiol reactivity and k_{cat}/K_m (Brocklehurst & Little, 1970, 1972; Sluysteran & Wijdenes, 1973; Shipton *et al.*, 1975; Lewis *et al.*, 1978; Brocklehurst & Malthouse, 1980; Salih *et al.*, 1987), and was postulated to play a

central role in the UP-DOWN mechanism put forward by Angelides & Fink (1978, 1979a,b) by which the catalytic site was considered to undergo substantial reorganization to meet postulated stereoelectronic requirements (Brocklehurst & Malthouse, 1978). Argument against the catalytically active 'DOWN conformation' of papain stabilized in its new grossly rotated position by interaction of the catalytic-site $-ImH^+$ with both $-S^-$ and $-CO_2^-$ is provided by the crystallographic evidence [see Brocklehurst *et al.* (1981) for a discussion]. In particular, the side chain of Asp-158 is turned away from the catalytic site (0.75 nm from $-S^-$ and 0.55 nm from $-ImH^+$), as is Asp-161 in actinidin, for which the 'UP conformation' exists in the crystal even though the crystals were grown at pH 6 where the $-S^-/-ImH^+/-CO_2^-$ ionization state predominates (Baker, 1981; Kamphuis *et al.*, 1985; Baker & Drenth, 1987). In each enzyme the carboxylate anion accepts a hydrogen bond from the main-chain NH of an alanine residue (Ala-136 in papain and Ala-139 in actinidin). The differences in the pH-dependent behaviour of actinidin and papain (see the Introduction) could arise from the ionization of analogous side chains if different micro-environments in the two enzymes provide them with different pK_a values. The idea that this could apply to the side chains of Asp-158 and Asp-161 in papain and actinidin respectively was suggested by Brocklehurst *et al.* (1983) and is supported by the calculations reported by Pickersgill *et al.* (1989). There is, however, no generality of influence of residues in this position in the cysteine proteinases. In some members of the family the analogous sequence-aligned residues (see Kamphuis *et al.*, 1985) do not contain carboxy groups, as is the case for cathepsin B (glycine), cathepsin H (asparagine) and

Scheme 2. Schematic drawing showing the different electrostatic influences to which the catalytic sites of actinidin and papain are exposed

(a) Actinidin at pH approx. 7: the catalytic-site ion-pair (Cys-25/His-162) is exposed to the electrostatic influence of Asp-161, which in turn is influenced by the proximity of Asp-138 [see Pickersgill *et al.* (1989) for a discussion of interaction energies]. The carboxylate anion of Asp-161 accepts a hydrogen bond from the backbone N-H of Ala-139, and Asp-138 is in a relatively solvent-accessible environment. Pickersgill *et al.* (1989) suggest that Asp-161 may have pK_a approx. 5.5 and be responsible for the pH-dependent kinetic behaviour of actinidin at pH values around 5.5 (see Salih *et al.*, 1987). Ala-159 is analogous to Lys-156 in papain (see below). (b) Papain at pH approx. 7: the catalytic site ion pair (Cys-25/His-159) is exposed to the electrostatic influence of Asp-158, but, as pointed out by Pickersgill *et al.* (1989), the additional influence of Glu-135 will be much less than the influence of Asp-138 on Asp-161 in actinidin because Glu-135 forms an ion-pair with Lys-156. Thus the pK_a of Asp-158 in papain might be expected to be lower than that of Asp-161 in actinidin, and the ionization of these analogous side chains in different microenvironments might be responsible for the kinetically influential pK_a values of 4 and 5.5 in papain and actinidin respectively (Brocklehurst *et al.*, 1983; Pickersgill *et al.*, 1989). In principle the dehydronation of the $-NH_3^+$ of Lys-156 could influence the behaviour of the Cys-25/His-159 ion-pair by releasing the carboxylate anion of Glu-135. It seems probable, however, that the pK_a of Lys-156 will be above 10, particularly in view of the stabilization provided by the proximity of Glu-135. The fraction of the system containing the ionic species shown in (c) will probably be very small, therefore, at any pH, and it seems unlikely that Lys-156 could contribute one of the two pK_a values (8.2 and 9.5) that control the behaviour of papain in alkaline media (Brocklehurst *et al.*, 1988b).

stem bromelain (asparagine). In actinidin the proximity (0.38 nm) of the carboxy groups of Asp-161 and Asp-138 (see Scheme 2) suggests that one of them might have a relatively high pK_a value. Experimental evidence of perturbation resulting from the proximity of two carboxy groups is provided by studies on Asp-52 and Glu-35 in lysozyme. The side chain of Glu-35 lies in a hydrophobic region near the bottom of the active-centre cleft of the enzyme (Blake *et al.*, 1967). This would be expected to provide for a raised pK_a value as a result of anion destabilization, and Dahlquist & Raftery (1968) assigned to Glu-35 the pK_a of 6.1 in lysozyme determined by n.m.r. measurements on pH-dependent binding of 2-deoxy-2-acetamido- β -D-glucopyranose. The electrostatic contribution to the raised pK_a of Glu-35 provided by the proximity of Asp-52 was estimated to be approx. 0.8 pK_a unit by difference potentiometry with native lysozyme and lysozyme with Asp-52 specifically esterified (Parsons & Raftery, 1970). In actinidin the interaction energy for the Asp-161/Asp-138 pair calculated by the method discussed by Pickersgill (1988) suggests an abnormally high pK_a value for one component, probably Asp-161 (Pickersgill *et al.*, 1989), since Asp-138 is in a more solvent-accessible environment. In papain the micro-environment of Asp-158 is different. The closest approach of Asp-158 and Glu-135 is 0.60 nm and the interaction energy is less than 50 % of that for the Asp-161/Asp-138 pair in actinidin (Pickersgill *et al.*, 1989). In addition, Pickersgill *et al.* (1989) pointed out that Glu-135 could form an ion-pair with Lys-156 (which is Ala-159 in actinidin). The possibility that the proximity of Lys-156 to the catalytic site of papain might perturb its characteristics was suggested by Malthouse & Brocklehurst (1978) in an attempt to account for the difference in properties between papain and ficin (EC 3.4.22.3), which, like actinidin, has a non-cationic residue (serine) in the position analogous to Lys-156 in papain. At that time, however, residue 135 in papain was thought to be glutamine, and it was only after the report of the nucleotide sequence (Cohen *et al.*, 1986) that the anionic nature of residue 135 (Brocklehurst *et al.*, 1989) and hence the possibility of ion-pair formation with Lys-156 (Pickersgill *et al.*, 1989) was noted. Although the existence of the Lys-156/Glu-135 ion-pair in papain, which is without analogue in actinidin (see Scheme 2), could account for a kinetically influential pK_a of 4 in papain but not in actinidin and for the more effectively influential pK_a of 5.5 in actinidin, it seems unable to account for either of the pK_a values of 8.2 and 9.5 that control the behaviour of papain in alkaline media discussed by Brocklehurst *et al.* (1988b).

Concluding comment

Identification of the groups responsible for the complex pH-dependent kinetic behaviour of actinidin probably requires an extensive series of protein-engineering experiments, and this analysis provides rational candidates for this type of study. The extensive electrostatic field to which the active-centre region of actinidin is exposed suggests that a multiplicity of charge effects may be kinetically influential, of which those associated with pK_a values 5 and 6.3 appear to play a central role when linked with particular binding interactions. The striking differences in reactivity characteristics between actinidin and papain despite closely similar folding patterns and virtually superimposable crystal structures in and around the

active-centre regions may involve similar binding-site-catalytic-site signalling mechanisms modulated differently by a variety of electrostatic effects.

We thank the Science and Engineering Research Council for project grants and for an Earmarked Studentship for D.K., Miss Liz Wilkie for skilled technical assistance, Miss Joy Smith for rapid production of the typescript and Professor Jan Drenth and Dr. Ted Baker for supplying a copy of the schematic drawing that was used to construct Scheme 1.

REFERENCES

- 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. (1980) *J. Mol. Biol.* **141**, 441–484
- Baker, E. N. (1981) in *Structural Studies on Molecules of Biological Interest* (Dodson, G., Glusker, J. P. & Sayre, D., eds.), pp. 339–349, Clarendon Press, Oxford
- Baker, E. N. & Drenth, J. (1987) in *Biological Macromolecules and Assemblies* (Jurnak, F. & McPherson, A., eds.), vol. 3, pp. 313–368, John Wiley and Sons, New York
- Berger, A. & Schechter, I. (1970) *Philos. Trans. R. Soc. London* **B 257**, 249–264
- Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C. & Sarma, V. R. (1967) *Proc. R. Soc. London* **B 167**, 378–388
- Brocklehurst, K. (1986) in *Cysteine Proteinases and their Inhibitors* (Turk, V., ed.), pp. 307–325, Walter de Gruyter, Berlin
- Brocklehurst, K. (1987) in *Enzyme Mechanisms* (Page, M. I. & Williams, A., eds.), pp. 140–158, Royal Society of Chemistry, London
- Brocklehurst, K. & Little, G. (1970) *FEBS Lett.* **9**, 113–116
- Brocklehurst, K. & Little, G. (1972) *Biochem. J.* **128**, 471–474
- Brocklehurst, K. & Malthouse, J. P. G. (1978) *Biochem. J.* **175**, 761–764
- Brocklehurst, K. & Malthouse, J. P. G. (1980) *Biochem. J.* **191**, 707–718
- Brocklehurst, K., Baines, B. S. & Malthouse, J. P. G. (1981) *Biochem. J.* **197**, 739–746
- Brocklehurst, K., Mushiri, S. M., Patel, G. & Willenbrock, F. (1983) *Biochem. J.* **209**, 873–879
- Brocklehurst, K., Salih, E. & Lodwig, T. S. (1984) *Biochem. J.* **220**, 609–612
- Brocklehurst, K., Kowlessur, D., O'Driscoll, M., Patel, G., Quenby, S., Salih, E., Templeton, W., Thomas, E. W. & Willenbrock, F. (1987a) *Biochem. J.* **244**, 173–181
- Brocklehurst, K., Willenbrock, F. & Salih, E. (1987b) in *Hydrolytic Enzymes* (Neuberger, A. & Brocklehurst, K., eds.), pp. 39–158, Elsevier, Amsterdam
- Brocklehurst, K., Kowlessur, D., Patel, G., Templeton, W., Quigley, K., Thomas, E. W., Wharton, C. W. & Willenbrock, F. (1988a) *Biochem. J.* **250**, 761–772
- Brocklehurst, K., Brocklehurst, S. M., Kowlessur, D., O'Driscoll, M., Patel, G., Salih, E., Templeton, W., Thomas, E. W., Topham, C. M. & Willenbrock, F. (1988b) *Biochem. J.* **256**, 543–555
- Brocklehurst, K., O'Driscoll, M., Kowlessur, D., Phillips, I. R., Templeton, W., Thomas, E. W., Topham, C. M. & Wharton, C. W. (1989) *Biochem. J.* **257**, 309–310
- Brocklehurst, S. M. & Brocklehurst, K. (1988) *Biochem. J.* **256**, 556–558
- Carne, A. & Moore, C. H. (1978) *Biochem. J.* **173**, 73–83

- Cohen, L. W., Coghlan, V. M. & Dihel, L. C. (1986) *Gene* **48**, 219–227
- Dahlquist, F. W. & Raftery, M. A. (1968) *Biochemistry* **7**, 3277–3289
- Finney, J. L. (1977) *Philos. Trans. R. Soc. London B* **278**, 3–32
- Kamphuis, I. G., Drenth, J. & Baker, E. N. (1985) *J. Mol. Biol.* **182**, 317–329
- Lavery, R., Pullman, A. & Wen, Y. K. (1983) *Int. J. Quantum Chem.* **24**, 352–371
- Lewis, S. D., Johnson, F. A., Ohno, A. K. & Shafter, J. A. (1978) *J. Biol. Chem.* **253**, 5080–5086
- Lowe, G. & Yuthavong, Y. (1971) *Biochem. J.* **124**, 107–115
- 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
- McDowall, M. A. (1970) *Eur. J. Biochem.* **14**, 214–221
- Parsons, S. M. & Raftery, M. A. (1970) *Biochem. Biophys. Res. Commun.* **41**, 45–49
- Pickersgill, R. W. (1988) *Protein Eng.* **2**, 247–248
- Pickersgill, R. W., Goodenough, P. W., Sumner, I. G. & Collins, M. E. (1988) *Biochem. J.* **254**, 235–238
- Pickersgill, R. W., Sumner, I. G., Collins, M. E. & Goodenough, P. W. (1989) *Biochem. J.* **257**, 310–312
- Salih, E. & Brocklehurst, K. (1983) *Biochem. J.* **213**, 713–718
- Salih, E., Malthouse, J. P. G., Kowlessur, D., Jarvis, M., O'Driscoll, M. & Brocklehurst, K. (1987) *Biochem. J.* **247**, 181–193
- Shipton, M., Kierstan, M. P. J., Malthouse, J. P. G., Stuchbury, T. & Brocklehurst, K. (1975) *FEBS Lett.* **50**, 365–368
- Sluyterman, L. A. Æ. & Wijdenes, J. (1973) *Biochim. Biophys. Acta* **302**, 95–101

Received 7 September 1988/7 November 1988; accepted 11 November 1988