Investigation of the catalytic site of actinidin by using benzofuroxan as a reactivity probe with selectivity for the thiolate–imidazolium ion-pair systems of cysteine proteinases

Evidence that the reaction of the ion-pair of actinidin (pK_I 3.0, pK_{II} 9.6) is modulated by the state of ionization of a group associated with a molecular pK_a of 5.5

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1. Benzofuroxan reacts with the catalytic-site thiol group of actinidin (EC 3.4.22.14, the cysteine proteinase from *Actinidia chinensis*) to produce stoichiometric amounts of the chromophoric reduction product, *o*-benzoquinone dioxime, and of a catalytically inactive derivative of actinidin that is devoid of thiol and that is assumed to contain, initially at least, the sulphenic acid of cysteine-25. A similar result applies also to papain (EC 3.4.22.2). 2. The rate of *o*-benzoquinone dioxime formation is neither increased by inclusion of 2-mercaptoethanol or hydroxylamine in the reaction mixture nor decreased by changing the solvent from H_2O to 2H_2O . The change of solvent was shown to be without effect also on the rate of reaction of benzofuroxan with papain. 3. These results suggest that the reactions of benzofuroxan with both actinidin and papain involve rate-determining attack of the catalytic-site thiol group to produce an intermediate adduct that then reacts rapidly with water to form enzyme sulphenic acid and *o*-benzoquinone dioxime. 4. The pH-dependence of the second-order rate constant for the reaction of benzofuroxan with actinidin was determined in the pH range 4.3–10.2. In marked contrast with the analogous reaction of papain {reported by Shipton & Brocklehurst [(1977) *Biochem. J.* 167, 799–810]} the pH– k profile for the actinidin reaction clearly contains a sigmoidal component with pK_a 5.5, in which k increases with decreasing pH. These data together with the molecular pK_a values for S^-/ImH^+ ion-pair formation and decomposition (3.0 and 9.6) suggest that the combined nucleophilic–electrophilic reactivity of the ion-pair of actinidin might be controlled by the state of ionization of another ionizing group, associated with the molecular pK_a of 5.5. The pH-dependence of k for the reaction of actinidin with benzofuroxan at 25°C at I 0.1 in aqueous buffers containing 6.7% (v/v) ethanol is probably adequately described by:

$$k = \tilde{k}_1 / (1 + [H^+]/K_I + K_{II}/[H^+]) + \tilde{k}_2 / (1 + [H^+]/K_{II} + K_{III}/[H^+]) + \tilde{k}_3 / (1 + [H^+]/K_{III})$$

in which $\tilde{k}_1 = 2.55 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\tilde{k}_2 = 1.35 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\tilde{k}_3 = 0.93 \text{ M}^{-1} \cdot \text{s}^{-1}$, $pK_I = 3.0$, $pK_{II} = 5.5$ and $pK_{III} = 9.6$. By contrast, the analogous reaction of papain may be described by the same equation but with $\tilde{k}_1 = 0$, $\tilde{k}_2 = 2.2 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\tilde{k}_3 = 1.3 \text{ M}^{-1} \cdot \text{s}^{-1}$, $pK_{II} = 3.6$ and $pK_{III} = 9.0$.

The possibility that the cysteine proteinases might provide an opportunity to study the effect of differences in active-centre structure on enzyme catalytic activity and on the reactivities of active-centre nucleophiles has been discussed previously (Brocklehurst *et al.*, 1983a). Two obvious targets for comparative study are papain (EC 3.2.22.2) and

actinidin (EC 3.4.22.14 the cysteine proteinase from kiwifruit, *Actinidia chinensis*). Papain has long been regarded as archetypal of cysteine proteinases, although evidence is accumulating that there are considerable differences between the active centres of the various cysteine proteinases and that papain, far from being archetypal, may actually possess some

properties not shared by any of the other cysteine proteinases studied to date. Studies on papain are supported by a considerable literature [for reviews see Lowe (1976), Brocklehurst *et al.* (1981a) and Polgár & Halász (1982)], and the crystal structure at 0.28 nm resolution (Drenth *et al.*, 1968, 1976) has been reported. The literature on actinidin is much less extensive, but the crystal structure at 0.17 nm resolution (Baker, 1980) has been reported. Although the catalytic sites of papain and actinidin appear to be very similar on the basis of the X-ray-crystallographic studies, differences that may be mechanistically significant have been deduced by using thiol-specific reactivity probes. Thus the apparent molecular pK_a values that control the formation and decomposition of the interactive systems that contain nucleophilic sulphur in neutral and acidic media appear to be more widely separated in actinidin (pK values approx. 3 and 10) than in papain (pK approx. 4 and 9) (Brocklehurst *et al.*, 1981b). Also, actinidin appears to bind the anionic probe 5,5'-dithiobis-(2-nitrobenzoate) dianion (Nbs_2^{2-}) relatively strongly, whereas papain repels it, the difference in the rates of its reaction with the thiolate anions of the two enzymes being approx. 1×10^3 (Brocklehurst *et al.*, 1983a).

The present paper reports a kinetic study of the reaction of the thiol group of actinidin with benzofuroxan. This reactivity probe reacts with the active-centre thiol groups of papain, ficin (EC 3.4.22.3) and bromelain (EC 3.4.22.4) by a mechanism that appears to allow the catalytically competent ion-pairs to react more rapidly than the simple thiolate anions (Shipton & Brocklehurst, 1977).

The reactivity of the actinidin thiol group is modulated by the state of ionization of an additional group associated with a molecular pK_a approx. 5.5, and this provides a profile of second-order rate constant (k) versus pH quite different in shape from those that characterize the reactions of benzofuroxan with papain, ficin and bromelain. The absence of effect on the rate of reaction of actinidin with benzofuroxan of addition of hydroxylamine to the reaction mixture or of replacement of solvent water by 2H_2O suggests that breakdown of the postulated covalent enzyme-reagent adduct is not rate-determining, a conclusion that applies also to the reaction of papain with benzofuroxan.

Materials and methods

Materials

Fully active actinidin containing 1 mol of thiol/mol of protein was prepared from frozen kiwifruit as described by Brocklehurst *et al.* (1981b).

Fully active papain was prepared from spray-dried papaya latex [known to have been produced

by the Boudart (1969, 1970) process in Zaire and kindly supplied by Powell and Scholefield, Liverpool, U.K.] as described by Baines & Brocklehurst (1979). Some types of commercially available latex provide a reasonably concentrated solution containing cysteine proteinases when mixed with water, but leave all of the papain in residual insoluble granules. Papain can be released from these granules by grinding them with sand. It is advisable to check the thiol content and reactivity characteristics of the product towards 2,2'-dipyridyl disulphide in both acidic (pH approx. 4) and alkaline (pH approx. 8) media to ensure that they conform to those of papain (see Baines & Brocklehurst, 1979). Alternatively, or preferably additionally, preparations should be subjected to analytical chromatography on SP-(sulphopropyl)-Sephadex-50 as described by Baines & Brocklehurst (1979), but with an elution gradient from 0.1 M-sodium acetate buffer, pH 5.0, to the same buffer containing 0.6 M-NaCl.

The synthesis and the characterization of benzofuroxan were described by Shipton *et al.* (1977). *N*- α -Benzoyl-L-arginine *p*-nitroanilide was obtained from Sigma Chemical Co. Hydroxylammonium chloride (AnalaR) and 2H_2O (spectrosol, minimum isotopic purity 99.75%) were obtained from BDH Chemicals.

For experiments in 2H_2O , solid buffer salts were dissolved directly in 2H_2O . A solution of benzofuroxan in spectroscopic-grade ethanol (5 ml of 15–30 mM) was diluted with 2H_2O to provide 12.5 ml of stock solution. In kinetic experiments 0.5 ml of the stock solution was diluted 6-fold to give a reaction mixture in 2H_2O containing 6.7% (v/v) ethanol. A stock solution of actinidin in 2H_2O was obtained as follows. Activated activator-free actinidin (10 ml) was prepared as described by Brocklehurst *et al.* (1981b), but with double the amount of kiwifruit pulp. This solution was concentrated by using an Amicon concentrator (50 ml) and Diaflo ultrafiltration membranes (43 mm) for 10 min and an N_2 pressure of 0.2 MPa (30 lbf/in²). Values of p^2H were calculated by adding 0.4 to the reading of the pH-meter (glass electrode) (Glascoe & Long, 1960).

Kinetics

The reactions of actinidin and papain with benzofuroxan were studied at 25°C at 10.1 in aqueous buffers (prepared as described by Shipton *et al.*, 1977) containing 6.7% (v/v) ethanol by monitoring the increase in absorbance at 416 nm, the isosbestic point in the pH-dependent spectrum of the product, *o*-benzoquinone dioxime, with a Cary 118C spectrophotometer and assuming $\epsilon_{416} = 5110 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Shipton *et al.*, 1977). The concentrations of enzyme and benzofuroxan were 1.5–3 μM and 0.5–3 mM respectively. The absorbance changes (approx. 0.01) in these reactions are

readily recorded by using the 0–0.024 range of the Cary 118C spectrophotometer. In some experiments hydroxylamine or 2-mercaptoethanol was included in the reaction mixtures.

Catalytic activity in the absence and in the presence of 10 mM-L-cysteine was measured at pH values 4.0 and 8.0 by using 1–3 mM-*N*- α -benzoyl-L-arginine *p*-nitroanilide as substrate, spectral analysis at 412 nm and $\Delta\epsilon_{410} = 8.8 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ as described by Malthouse & Brocklehurst (1976).

Construction of the pH-*k* profiles for the reactions of benzofuroxan with actinidin and with papain

The lines of Fig. 1 were constructed from data, generated by using eqns. (1) and (2) and a BBC Microcomputer (model B), for pH values 0–12 at intervals of 0.2 pH unit. For the actinidin reaction the line had been shown to fit the experimental values collected in the pH range 4.3–10.2 by superimposing on the visual-display unit the data points (shown in Fig. 1) on a line generated by using 0.01 intervals of pH. Eqn. (1) describes the pH-dependence of *k* for a reaction in three protonic states characterized by pH-independent rate constants $\tilde{k}_1$ – $\tilde{k}_3$ and molecular $\text{p}K_a$ values $\text{p}K_I$ – $\text{p}K_{III}$ (see Brocklehurst, 1974):

$$k = \tilde{k}_1 / (1 + [\text{H}^+]/K_I + K_{II}/[\text{H}^+] + K_{II}K_{III}/[\text{H}^+]^2) + \tilde{k}_2 / (1 + [\text{H}^+]^2/K_I K_{II} + [\text{H}^+]/K_{II} + K_{III}/[\text{H}^+]) + \tilde{k}_3 / (1 + [\text{H}^+]^3/K_I K_{II} K_{III} + [\text{H}^+]^2/K_{II} K_{III} + [\text{H}^+]/K_{III}) \quad (1)$$

$$k = \tilde{k}_1 / (1 + [\text{H}^+]/K_I + K_{II}/[\text{H}^+]) + \tilde{k}_2 / (1 + [\text{H}^+]/K_{II} + K_{III}/[\text{H}^+]) + \tilde{k}_3 / (1 + [\text{H}^+]/K_{III}) \quad (2)$$

Eqn. (2) is an approximate form of eqn. (1), and it applies when the three protonic dissociations are well separated from each other. Then the contributions containing multiple powers of $[\text{H}^+]$ may be neglected. The superimposition on the visual-display unit of the lines corresponding to eqns. (1) and (2) for the actinidin reaction with $\text{p}K_I = 3.0$, $\text{p}K_{II} = 5.5$, $\text{p}K_{III} = 9.6$, $\tilde{k}_1 = 2.55 \text{ M}^{-1} \cdot \text{s}^{-2}$, $\tilde{k}_2 = 1.35 \text{ M}^{-1} \cdot \text{s}^{-1}$

and $\tilde{k}_3 = 0.93 \text{ M}^{-1} \cdot \text{s}^{-1}$ showed the validity of the approximation in this case. The difference between values of *k* calculated by using the two equations was always less than 0.5%. It was convenient to write the computer program in index form so that, for example, the last term in eqn. (2) was input as $\tilde{k}_3/[1 + (10^{-\text{pH}})(10^{\text{p}K_{III}})]$. For papain reaction, which displays reactivity in only two protonic states, $\tilde{k}_1$ was set equal to zero. The values of the other parameters are the optimized values reported by Shipton & Brocklehurst (1977).

Results and discussion

Benzofuroxan as a reactivity probe for the study of the nucleophilic characteristics of the ion-pairs in the catalytic sites of cysteine proteinases

Benzofuroxan (benzofurazan 1-oxide, benzo-2-oxa-1,3-diazole *N*-oxide) (I, Scheme 1) reacts with thiol groups in simple molecules of low M_r (RSH) to produce *o*-benzoquinone dioxime (II, Scheme 1) and the corresponding disulphide (R–S–S–R) (Shipton *et al.*, 1977). The reaction is first-order with respect to concentration not only in benzofuroxan but also in RSH, despite the fact that the disulphide, R–S–S–R, is formed (Shipton & Brocklehurst,

1977). This finding can be accommodated by postulating the rate-determining formation of an intermediate adduct of RSH and benzofuroxan in 1:1 stoichiometry followed by rapid reaction of this adduct with another molecule of RSH. The pH-dependence of the reaction (a pH-*k* profile of simple sigmoidal form, *k* increasing with increasing pH) is in accord with rate-determining attack of RS^- at one

Scheme 1. Reactions of benzofuroxan (I) with thiol groups in (a) simple molecules of low M_r (RSH) and (b) some cysteine proteinases (ESH)

In both cases the chromophoric product *o*-benzoquinone dioxime (II) is formed. In reactions of RSH, the disulphide is formed. In reactions of ESH a catalytically inactive derivative devoid of thiol is formed, which is probably the corresponding sulphenic acid and/or sulphinic acid.

of the three electrophilic centres of benzofuroxan (N-3, C-4 or C-6) followed by post-transition-state protonation to form an adduct such as (III) (in which nucleophilic attack is shown as having occurred at C-6). Subsequent rapid attack on the S atom of the adduct by another molecule of RS^- would lead to the disulphide and dioxime products.

The reactions of benzofuroxan with the catalytic-site thiol groups of papain, ficin and bromelain differ in both stoichiometry and pH-dependence from its reactions with low- M_r thiols (Shipton & Brocklehurst, 1977). Thus 1 mol of enzyme (thiol) reacts with 1 mol of benzofuroxan to produce 1 mol of *o*-benzoquinone dioxime and 1 mol of catalytically inactive enzyme. Also, the pH- k profiles are not of simple sigmoidal form, but rather contain striking bell-shaped components with rate optima around pH 6 as well as lower plateau rates at high pH. This shape suggests that, if the rate-determining step is again adduct formation, the attack of the thiolate anion of the catalytically competent ion-pair of the enzyme might be more effective than the attack of the thiolate ion when the catalytic-site imidazolium ion is deprotonated. A further complexity exists, however, because of the probable two-step nature of the covalency changes in the reaction of this probe. Thus another possibility is that an adduct could form rapidly and reversibly in low steady state, e.g. by reaction at N-3 of benzofuroxan, and that the pH-dependence might reflect attack of water at the S atom of the adduct, base-catalysed by the imidazole group of the catalytic-site histidine residue. This transition state would contain the proton stoichiometry required to account for the bell-shaped component of the pH- k profile, i.e. S^- , Im and one proton. In the enzyme reactions, where intermolecular disulphide formation is excluded on steric grounds, and by the reaction stoichiometry, the catalytically inactive enzyme derivative is considered to be one of the oxy acid oxidation products, initially the sulphenic acid as shown in Scheme 1. In an attempt to ascertain whether adduct formation of adduct breakdown is rate-determining in the reactions of benzofuroxan with papain and with actinidin, the reactions were performed (a) in the presence of added nucleophiles (hydroxylamine and 2-mercaptoethanol separately) and (b) in $^2\text{H}_2\text{O}$. These reactions were performed in

approximately neutral media, where the rates of reaction of benzofuroxan with papain (Shipton & Brocklehurst, 1977) and with actinidin (see below) are insensitive to change in pH. The results shown in Table 1 provide no evidence that the rates of *o*-benzoquinone dioxime formation are affected by any of the added nucleophiles, which might have been expected to change the rate of reaction if adduct breakdown were rate-determining. These results, particularly the lack of effect on k of changing the solvent from H_2O to $^2\text{H}_2\text{O}$, suggest that adduct formation is rate-determining in the enzyme reactions as it is in the reactions of simple low- M_r thiols. The rate optimum around pH 6–7 in the papain reaction is probably best interpreted, therefore, in terms of binding of ImH^+ of the ion-pair to the *N*-oxide oxygen of benzofuroxan and attack of the thiolate anion of the ion-pair at C-6. The pH- k profile for the reaction of papain with the alkylating agent 1-bromo-4-hydroxy-3-nitroacetophenone (IV) (Furlanetto & Kaiser, 1973) is similar in shape to that for the reaction of papain with benzofuroxan.

Inspection of structures (I) and (IV) does not reveal an exact analogy in terms of relative dispositions of the N^+-O^- group and the electrophilic centre attacked. There may be sufficient similarity, however, and sufficient flexibility in the enzyme-reagent complexes for binding of the rather flexible reagent (IV) to mimic sufficiently the binding of the rigid reagent (I). The relatively high rates of reaction of the ion-pair of papain towards the two reagents therefore may have similar cause.

It appears to be reasonable to conclude that benzofuroxan may be a useful reactivity probe to study the reactivity characteristics of the ion-pairs of

Table 1. Effects of $^2\text{H}_2\text{O}$ and of other added nucleophiles on the second-order rate constants (k) for *o*-benzoquinone dioxime formation in reactions of benzofuroxan with the thiol groups of papain and actinidin at 25°C at $I\ 0.1$

In the pH range 6.5–8, the rates of the reactions of benzofuroxan with both enzymes are essentially pH-independent.

Solvent	pH or $p^2\text{H}$	Added nucleophile	Enzyme	$k\ (\text{M}^{-1}\cdot\text{s}^{-1})$
H_2O	7.13	—	Papain	1.95
H_2O	7.13	—	Papain	2.00
$^2\text{H}_2\text{O}$	7.70	—	Papain	1.79
$^2\text{H}_2\text{O}$	7.68	—	Papain	1.85
H_2O	7.18	—	Actinidin	1.43
H_2O	7.19	—	Actinidin	1.47
$^2\text{H}_2\text{O}$	7.67	—	Actinidin	1.51
$^2\text{H}_2\text{O}$	7.64	—	Actinidin	1.47
H_2O	7.13	$17\ \mu\text{M}$ -2-Mercaptoethanol	Actinidin	1.39
H_2O	6.79	$12.5\ \text{mM}$ - NH_2OH	Actinidin	1.26

cysteine proteinases by using a reaction in which the ion-pair is the more reactive enzyme form containing thiolate anion, as in the catalytic act itself, and that is readily studied by continuous spectral analysis. The fact that the covalency change is multi-step in nature is not a complicating factor if adduct formation is rate-determining. The rate of *o*-benzoquinone dioxime formation then reflects the reactivity characteristics of the two nucleophilic states of the enzyme thiol group (S^-/ImH^+ and S^-/Im).

Kinetics of the reaction of benzofuroxan with the thiol group of actinidin

In the pH range 4.3–10.2 benzofuroxan (0.5–3 mM) reacts with actinidin (1.5–3 μM) to produce *o*-benzoquinone dioxime stoichiometric with the actinidin thiol group. The reactions followed first-order kinetics with respect to time, as assessed by the linearity of conventional logarithmic plots, to at least 80% reaction, and the linearity of plots of first-order rate constant against benzofuroxan concentration establishes that the reaction is also first-order with respect to concentration in benzofuroxan (at least up to 3 mM).

Actinidin that had been allowed to react fully with excess of benzofuroxan at pH values 4.1 and 8.3 was devoid of catalytic activity towards *N*- α -benzoyl-L-arginine *p*-nitroanilide. Samples of inactivated actinidin (pH values 4.1 and 8.3) that had been left to stand overnight and then freed from excess of benzofuroxan by chromatography through Sephadex G-25 were not re-activated by addition of 10 mM-L-cysteine when assayed with *N*- α -benzoyl-L-arginine *p*-nitroanilide at pH values 4.0 and 8.0.

pH-dependence of the rate of reaction of the actinidin thiol group with benzofuroxan

The pH-dependence of the apparent second-order rate constant (k) for the formation of *o*-benzoquinone dioxime in reaction mixtures containing actinidin and excess benzofuroxan is compared with the corresponding pH- k profile for the reaction of benzofuroxan with the papain thiol group in Fig. 1.

The reaction of benzofuroxan with papain (Shipton & Brocklehurst, 1977) appears to involve reaction in two ionization states, that of higher reactivity involving the S^-/ImH^+ ion-pair (produced by protonic dissociation with molecular pK_a 3.6 and converted into S^-/Im by protonic dissociation of pK_a 9) and that of lower reactivity involving the 'uncomplicated' thiolate anion (S^-/Im). Thus for the papain reaction the pH- k profile in Fig. 1 rises from a plateau value of k at high pH across a pK_a of 9 to an optimum value of k around pH 7, from which it falls (presumably to approach $k = 0$) across a pK_a of 3.6.

Fig. 1. pH-dependence of the second-order rate constants for the reactions of benzofuroxan with (a) actinidin and (b) papain at 25°C at 10.1 in aqueous buffers containing 6.7% (v/v) ethanol

The points are experimental for the actinidin reaction. The continuous and broken lines are both theoretical for eqn. (1) (and eqn. 2) of the Materials and methods section and the following values of the characterizing parameters. The values for the actinidin reaction (continuous line) are followed in parentheses by the values for the papain reaction (broken line): $\tilde{k} = 2.55(0) M^{-1} \cdot s^{-1}$; $\tilde{k}_2 = 1.35(2.2) M^{-1} \cdot s^{-1}$; $\tilde{k}_3 = 0.93(1.3) M^{-1} \cdot s^{-1}$; $pK_I = 3.0$ (not relevant); $pK_{II} = 5.5(3.6)$; $pK_{III} = 9.6(9.0)$. For the actinidin reaction pK_{II} and k_2 were obtained by inspection of the data and of the fitted line displayed on the visual-display unit of the computer; $\tilde{k}_1$ was similarly obtained by making use in addition of the value of pK_I deduced from studies with the two-protonic-state reactivity probe *n*-propyl 2-pyridyl disulphide (Brocklehurst *et al.*, 1981b), which also provided the value of pK_{III} ; the value of $\tilde{k}_3$ was estimated by using the values of $\tilde{k}_2$ and pK_{III} . The value of this parameter and that of $\tilde{k}_1$ were then refined on the visual-display unit by inspection of the fitted curve as minor adjustments to the values of the parameters were made. For the papain reaction, the broken line shown is the theoretical line corresponding to the parameters optimized from the experimental data reported by Shipton & Brocklehurst (1977). The arrows indicate the extent of the data collected for the papain reaction.

In marked contrast with the pH- k profile for the papain reaction, that for the reaction of benzofuroxan with actinidin contains a sigmoidal component in which k increases substantially as the pH is decreased from approx. 7.5 to approx. 4.5. In addition, k decreases as the pH is increased from 8.7 to approx. 10, which is similar to the change in the papain profile in alkaline media. Kinetic study of the reaction of benzofuroxan with actinidin was limited to the pH range 4.3–10.2 because of the instability of the enzyme outside these limits of pH over time scales required to study reactions with values of k approx. $1\text{--}2\text{ M}^{-1}\cdot\text{s}^{-1}$. It was possible, however, to construct the more extensive pH- k profile for the actinidin reaction shown in Fig. 1 by making use of the molecular pK_a values for the formation ($\text{ImH}^+/\text{SH} = \text{ImH}^+/\text{S}^- + \text{Im}/\text{SH}$; pK_a 3.0) and decomposition (to Im/S^- ; pK_a 9.6) of the enzyme state containing the ion-pair, deduced by using a highly reactive two-protonic-state reactivity probe (Brocklehurst *et al.*, 1981b). Combination of the data from the present study with the two pK_a values (3.0 and 9.6) deduced previously results in the pH- k profile for the actinidin reaction shown in Fig. 1. Thus at low pH the enzyme catalytic site exists mainly in the non-nucleophilic state ImH^+/SH . The nucleophilic state responsible for reactivity at pH values around 4.5 results from protonic dissociation (pK_a 3.0), which produces the nucleophilic ion-pair, ImH^+/S^- . The reaction of this ion-pair, however, appears to be modulated by the state of ionization of another group associated with molecular pK_a 5.5. When this group becomes proton-deficient, the reactivity of the ion-pair decreases to a new plateau value in the pH region approx. 7.5–8.5. Finally, the value of k decreases across the pK_a value of 9.6 as the ion-pair is converted by protonic dissociation into Im/S^- , and the assistance to the attack of thiolate ion on benzofuroxan provided by the imidazolium ion is thus lost.

It might be that a similar modulating group exists also in papain, but that its effect is not manifested in such an obvious way as it is in this reaction of actinidin, because of the closer similarity between its pK_a value and that characteristic of ion-pair formation. Overlapping protonic dissociations can result in apparently simple sigmoidal components in pH- k profiles when two ionization states of reaction have non-zero values (Brocklehurst *et al.*, 1983b). The acid limb of the pH- k profile for the papain reaction in Fig. 1, which appears to characterize a single ionization, could, in fact, result from two overlapping ionizations connecting a state of zero reactivity and two states of non-zero reactivity, as does the obviously more complex part of the pH- k

profile for the actinidin reaction between pH < 2 and pH 7.

It remains to be established whether the ionization in actinidin associated with pK_a 5.5 controls the intrinsic nucleophilic character of the thiolate ion of the ion-pair or whether its effect is on the assistance to the nucleophilic attack provided by association of the imidazolium ion with the electrophilic reagent. There is at present no evidence to suggest whether this modulating group is the carboxy group of aspartic acid-161 [see Baker (1980) for an account of the structure of actinidin] or a group remote from the immediate region of the catalytic site.

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