Variation in the pH-dependent pre-steady-state and steady-state kinetic characteristics of cysteine-proteinase mechanism: evidence for electrostatic modulation of catalytic-site function by the neighbouring carboxylate anion

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The acylation and deacylation stages of the hydrolysis of *N*-acetyl-Phe-Gly methyl thionoester catalysed by papain and actinidin were investigated by stopped-flow spectral analysis. Differences in the forms of pH-dependence of the steady-state and pre-steady-state kinetic parameters support the hypothesis that, whereas for papain, in accord with the traditional view, the rate-determining step is the base-catalysed reaction of the acyl-enzyme intermediate with water, for actinidin it is a post-acylation conformational change required to permit release of the alcohol product and its replacement in the catalytic site by the key water molecule. Possible assignments of the kinetically influential pK_a

values, guided by the results of modelling, including electrostatic-potential calculations, and of the mechanistic roles of the ionizing groups, are discussed. It is concluded that Asp¹⁶¹ is the source of a key electrostatic modulator (pK_a 5.0 ± 0.1) in actinidin, analogous to Asp¹⁵⁸ in papain, whose influence is not detected kinetically; it is always in the 'on' state because of its low pK_a value (2.8 ± 0.06).

Key words: actinidin, cysteine proteinase mechanism, electrostatic potential calculations, electrostatically modulated protein dynamics, papain; pH-dependent kinetics.

INTRODUCTION

We have demonstrated, using a variety of experimental approaches, the value of using natural variants of the papain family of cysteine proteinases to reveal gradations in functional characteristics and mechanistic phenomena that are more easily demonstrated in some variants than in others [1–4]. A recent study [5] provided evidence of a particularly marked difference in observed features of catalytic mechanism between papain (EC 3.4.22.2) and caricain [papaya (*Carica papaya*) proteinase Ω (EC 3.4.22.30)] on the one hand and actinidin (EC 3.4.22.14) and ficin (EC 3.4.22.3) on the other. Papain is the much studied minor cysteine proteinase component of the latex of *C. papaya* and caricain a major companion cysteine proteinase component of the same latex. Actinidin and ficin are cysteine proteinases from *Actinidia chinensis* (kiwi fruit or Chinese gooseberry) and, e.g., *Ficus glabrata* (matapalo or small-leaved fig) respectively. A combination of pre-steady-state and steady-state kinetic studies using a specific thionoester substrate established acyl-enzyme intermediate (ES') formation for ficin and actinidin to be reversible, a phenomenon that could not be demonstrated for either papain or caricain. These results, taken together with those of different experiments on ficin reported by Hollaway and Hardman [6] (see below), suggest a hypothesis in which the isomerization of the acyl-enzyme–alcohol product complex

(ES' · P₁) to a conformer that allows rapid release of P₁ into bulk solvent is rate-determining.

These events may be represented by a five-step model. Eqn (1), in which ES is the adsorptive complex and (ES' · P₁)* represents the conformation of the complex from which the alcohol diffuses rapidly to leave (ES')*. This is followed by binding of water and deacylation to regenerate the enzyme, with the formation of the carboxylate product, P₂.

The minimal model of catalysis of the hydrolysis of an ester (or amide) substrate by a cysteine proteinase (E) (eqn 2) involves simultaneous formation of ES' and the alcohol or amine (P₁) by reaction within an adsorptive complex (ES) followed by deacylation (hydrolysis) of ES'.

Evidence for the existence of ES and ES', tetrahedral species between ES and ES' and between ES' and E + P₂, and electrostatic

Abbreviations used: ABNR, adopted basis Newton–Raphson; Z, benzyloxycarbonyl; Im, imidazole; Im⁺H, imidazolium; MD, molecular dynamics; m.p., melting point; Nbf-Cl, 4-chloro-7-nitrobenzofurazan; SF, stopped-flow.

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Figure 1 The specific thionoester substrate (1) used in the kinetic investigation of cysteine-proteinase mechanism and the corresponding acyl-enzyme intermediates (2a and 2b)

modulation of catalytic-site geometry required to promote the acylation reaction is discussed in [1–4] and summarized in [5]. Additional modulation by specific binding interactions is discussed, e.g., in [7]. Most recently we reported evidence for the relatively long-lived $ES' \cdot P_1$ complexes in ficin- and actinidin-catalysed hydrolyses and for the reversibility of their formation [5]. This was achieved by direct observation of ES' using the new specific thionoester substrate *N*-acetyl-Phe-Gly methyl thionoester (structure 1, Figure 1). Thionoester substrates provide the opportunity to monitor the formation and subsequent hydrolysis of the dithioester moiety of the ES' component (such as structure 2, Figure 1) of the postulated $ES' \cdot P_1$ complex by spectral analysis at 315 nm. The possibility of observing ES' spectroscopically had been established by Lowe and Williams [8] in the case of papain and ficin by using methyl thionohippurate (benzoylglycine methyl thionoester). The investigation using compound 1 reported in [5] was prompted by the report from the study of ficin-catalysed hydrolysis by Hollaway and Hardman [6] that the release of 4-nitrophenol from benzyloxycarbonyl (Z)-Lys 4-nitrophenyl ester closely paralleled proton release monitored by using Chlorophenol Red as indicator. These results were interpreted as simultaneous release of both P_1 and P_2 into bulk solution, which suggests the additional steps in the catalytic model (eqn 1). If the k_{+3} step is rate-determining, this predicts the reversibility of the k_{+2} step established by the experiments reported in [5]. The differences observed in the kinetic behaviour of the enzyme variants towards the methyl thionoester substrate (1) might be due to the existence in actinidin, but not in papain or caricain, of the Asp¹⁴²–Lys¹⁴⁵ salt bridge. Molecular-dynamics (MD) simulations provided a possible explanation for the accumulation of the methanol (P_1) product in the actinidin reaction and, thus, for the observed reversibility of dithioacyl-enzyme formation [5]. Assistance by the catalytic-site imidazolium (Im^+H) cation is expected to result in the formation of methanol initially hydrogen-bonded to the neutral imidazole (Im) group. After minimization, the methanol was found, in models of all three enzymes, to donate a hydrogen bond to Asp¹⁵⁸ and to accept a hydrogen bond from His¹⁵⁹. During the subsequent

MD simulations the methanol was deduced to diffuse rapidly (over <8 ps) out of papain and caricain by various pathways. In marked contrast, the methanol molecule 'lingers' around the active centre of actinidin, particularly in the region encompassing Lys¹⁴⁵–His¹⁶² and Phe¹⁴⁴, Trp¹⁸⁴ and Trp¹⁸⁸. Uniquely in actinidin, Lys¹⁴⁵ makes a salt bridge with Asp¹⁴², the analogous interactions in papain (Lys¹³⁹ and Gln¹⁴²) and in caricain (Arg¹³⁹ and Gln¹⁴²) being much weaker. In actinidin the region between residues 140 and 146, together with Trp¹⁸⁴ and Trp¹⁸⁸, form a cavity which appears to entrap the methanol molecule. This could provide the possibility of reversing the acylation reaction by methanolysis of the dithioester intermediate deduced kinetically. Subsequently this cavity undergoes large scale movements [over approx. 4.8 Å (1 Å = 0.1 nm)] in a highly correlated manner which appear to control the motions of the methanol molecule. The changes in the cavity might be associated with the k_{+3} step in the model of eqn (1), which lead to rapid release of the methanol into bulk solvent associated with the k_{+4} step.

The thionoester substrate (1) was designed to contain the major recognition features of the papain family to provide a good fit in the S_2 -subsite and S_1 – S_2 intersubsite regions and a small, non-aromatic, non-electronically activated leaving group. In these respects it differs from Z-Lys-4 nitrophenyl ester, the substrate used by Hollaway and Hardman [6]. The design features of (1) and electrical neutrality were incorporated to obviate potential complications from electrostatic effects involving the substrate, a 'sticky' aromatic leaving group and electronically assisted leaving-group departure, and to provide for simple direct observation of the ES' moiety and the possible influence of key recognition features in the substrate.

A necessary consequence of the hypothesis that catalysis by papain may be described by the model of eqn (2), whereas catalysis by actinidin requires the extended model, eqn (1), is that the His-Im-assisted reaction of the acyl-enzyme intermediate with water is rate-determining for papain, but not for actinidin. Thus, for papain, hydrolysis of ES' would be characterized by k_{+3} of eqn (2), whereas, for actinidin, hydrolysis of the acyl-enzyme is characterized by k_{+5} of eqn (1) and the rate-determining step, characterized by k_{+3} of eqn (1), is the $ES' \cdot P_1 \rightarrow (ES' \cdot P_1)^*$ isomerization. This is followed by the two rapid steps (k_{+4} and k_{+5}) that result ultimately in the hydrolysis reaction. The work reported in [5] was carried out at pH 5.22. In the present paper we report evidence in favour of this hypothesis from pH-dependent kinetic studies using the same thionoester substrate (1). The results show, in particular, the predicted absolute dependence of k_{+3} on the proton-poor form of a low- pK_a ionizing group in papain, as required for general-base catalysis in the hydrolysis step (possibly with minor modulation by a neighbouring ionizing group), and that, by contrast, this is not the case for k_{+3} of the actinidin-catalysed hydrolysis. Rather, for actinidin, the pH- k_{+3} profile is a shallow sigmoid in which the value of k_{+3} is only little affected by the state of ionization of a low pK_a acid. The forms of pH-dependence of the steady state and pre-steady state kinetic parameters for both enzymes and the possible assignments of the kinetically influential pK_a values, guided by the results of electrostatic-potential calculations are discussed. It is particularly noteworthy that, whereas the pK_a values of Asp¹⁵⁸–CO₂H in papain and caricain are 2.8 and 2.0 respectively, the pK_a value of an analogous side chain in actinidin, within the Asp¹³⁸/Asp¹⁶¹ couple, is deduced to be associated with $pK_a \approx 5$. The consequence is that, whereas in papain and caricain the catalytic site is always under the electrostatic influence of Asp¹⁵⁸–CO₂ during the catalytic act, in actinidin the promotion of catalysis by the monoanion of the Asp¹³⁸/Asp¹⁶¹ couple is readily observed kinetically in the pH region 4–6.

MATERIALS AND METHODS

Enzymes and thionoester substrate (1)

Procedures for the purification of actinidin [9] and papain [10], which include those for the production of the fully active enzymes by covalent chromatography, have been described previously, as has their active-centre evaluation by spectroscopic titration at 343 nm ($\epsilon_{343} = 8080 \text{ M}^{-1} \cdot \text{cm}^{-1}$) using 2,2'-dipyridyl disulphide as titrant [11].

N-Acetyl-Phe-Gly methyl thionoester (compound **1**) was synthesized and characterized as described in [5] and further characterized by the NMR spectrum in DMSO.

^1H -NMR (250 MHz): δ 8.6 (t, 1 H, J 5 Hz, $\text{NH}-\text{CH}_2-\text{C}=\text{S}$), δ 8.1 (d, 1 H, J 8 Hz, $\text{NH}-\text{COCH}_3$), δ 7.3–7.1 (m, 5 H, aromatic), δ 4.6 (m, 1 H, $\text{NH}-\text{CH}-\text{C}=\text{O}$), δ 4.2 (dd, 1 H, J 16 Hz, 5 Hz, $\text{NH}-\text{CH}_2-\text{C}=\text{S}$), δ 4.1 (s, 3 H, $\text{CH}_3-\text{O}-\text{C}=\text{S}$), δ 4.05 (dd, 1 H, J 16 Hz, 5 Hz, $\text{NH}-\text{CH}_2-\text{C}=\text{S}$), δ 3.1 (dd, 1 H, J 12 Hz, 5 Hz, $\text{Ph}-\text{CH}_2-\text{CH}$), δ 2.75 (dd, 1 H, J 12 Hz, 10 Hz, $\text{Ph}-\text{CH}_2-\text{CH}$), δ 1.75 (s, 3 H, CH_3-CONH). This solvent provides high resolution and allows clear attribution of each signal.

The four-step synthesis of compound **1** involves in the last two steps reaction of a nitrile with methanol and HCl gas to provide an imidoester and reaction of this with H_2S in methanolic pyridine. It is important to point out that it is essential to use methanol and pyridine that have been thoroughly dried to ensure a pure product following recrystallization from ethyl acetate. Contamination of the product that results from inadequately dried solvents is readily discerned by inspection of the ^1H -NMR spectrum in DMSO and is not easily removed by recrystallization from ethyl acetate.

Kinetic studies using the thionoester substrate (1) and stopped-flow (SF) methodology

All reactions were carried out at 25 °C and I 0.1 in solutions of various pH values in the range ≈ 3.5 –8.5 containing 1 mM EDTA and aq. 4.2 % (v/v) DMSO, 100–300 μM thionoester substrate and 4.59–7.80 μM enzyme such that $[\text{S}]_0 > 10[\text{E}]_{\text{T}}$ (where $[\text{S}]_0$ is the initial substrate concentration and $[\text{E}]_{\text{T}}$ is the total enzyme concentration). Solvents were degassed under decreased pressure prior to preparation of reactant solutions. Kinetic studies were performed with an Applied Photophysics SF.17MV stopped-flow spectrophotometer, kinetics workstation and data-acquisition and analysis software. Monochromator entrance and exit slits were set at 1 mm. Formation and subsequent hydrolysis of the dithioacyl-enzyme intermediates were monitored at 315 nm. A_{315} -against-time (t) data were recorded over 50 s for the reactions of papain and actinidin in order to observe the rapid formation of the dithioacyl-enzyme intermediate and analyse its first-order decay to provide the first-order rate constant (k). The reactions were monitored also over 200 ms for papain and 1 s for actinidin, which isolates the increase in A_{315} in order to obtain the observed first-order rate constant (k_{obs}) for the formation of the dithioacyl-enzymes. The rate constants were evaluated by fitting the A_{315}/t data collected by the Acorn (Cambridge, Cambridgeshire, U.K.) Archimedes microcomputer of the SF apparatus to the equation for a single exponential process:

$$A = P_1 e^{-P_2 t} + P_3,$$

where

$$P_1 = |A_{\infty} - A_0|, P_2 = k_{\text{obs}} \text{ or } k, \text{ and } P_3 = A_{\infty}.$$

Subsequent processing of kinetic results was carried out by using Sigmaplot 5.0 (Jandel Scientific, Erkrath, Germany).

pH-dependence of $k_{\text{cat}}/K_{\text{m}}$ using N^{α} -benzoyl-L-arginine 4-nitroanilide as substrate

Initial rates of catalysis (v_i) by actinidin in 8 % (v/v) N,N -dimethylformamide, I 0.1 at 25 °C, were determined by spectral analysis at 410 nm ($\Delta\epsilon_{410} = 8.8 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$) as described previously [3] for the hydrolysis of another 4-nitroanilide catalysed by other cysteine proteinases. Values of $[\text{S}]_0 = 130 \mu\text{M}$ and $[\text{E}]_{\text{T}} = 3 \mu\text{M}$ provided the condition $[\text{S}]_0 \ll K_{\text{m}}$ ($K_{\text{m}} \leq 3 \text{ mM}$) which allows the calculation of $k_{\text{cat}}/K_{\text{m}}$ as $v_i/([\text{E}]_{\text{T}} \cdot [\text{S}]_0)$ at various pH values.

Computer evaluation of pH-dependent kinetic data

These were evaluated by a combination of curve-sketching and weighted non-linear regression as described in [3]. pH-dependent kinetic studies, sometimes involving a multiplicity of reactive states, require a rapid method of evaluating a series of kinetic models differing in the number and reactivity of the reactive states (see [12,13]). Such a method is provided by the multitasking application program SKETCHER [14,15]. SKETCHER permits rapid estimation of characterizing parameters in the generic set of equations for the various models by means of interactive manipulation of calculated curves. Values of the parameters thus obtained provide provisional estimates for analysis by weighted non-linear regression performed by using the non-linear regression program in Sigmaplot 5.0 (Jandel Scientific). The non-linear regression values are generally close to the SKETCHER values, particularly when one or more of the parameters is known from an independent experiment, which obviates problems deriving from multiple local minima.

Comparison of the electrostatic influences to which the active centres of actinidin and papain are exposed

The crystal structures of actinidin (2act.pdb [16]), papain (9pap.pdb [17]) and caricain (1ppo.pdb [18]) with the blocking group bonded to the catalytic-site CysS $^{\gamma}$ removed were used as the basis for modelling. The starting co-ordinates were taken from the Protein Data Bank [19]. The buried water molecules (25 in papain, 20 in actinidin and 24 in caricain) were retained. All residues were assumed to be in their fully charged states, that is, arginine, lysine and N-termini with charges of +1, aspartic acid, glutamic acid and C-termini with charges of -1, and the catalytic-site cysteine and histidine side chains with charges of -1 and +1 respectively, as required for the ion-pair state. The proteins were represented by the polar-hydrogen CHARMM (Chemistry at HARvard Macromolecular Mechanics) force field [20,21] and the water molecules by the transferable intermolecular potential ('TIP3') [22]. The initial treatment of the structures was carried out as described for our modelling of two thiolsubtilisins [23]. The non-bonded interactions were truncated at 14 Å and the electrostatic interactions at 12 Å and truncation of the van der Waals interactions was initiated at 8 Å and was complete by 12 Å. The substrate co-ordinates were constructed by using the EDITOR module of QUANTA (Molecular Simulations, San Diego, CA, U.S.A.), and electron densities were assigned by using the charge template module of QUANTA.

For papain and actinidin the substrate was docked as the dithioacyl-enzyme intermediate with the sulphur atom of Cys 25 bonded to the electrophilic carbon atom of the acyl moiety of the thionoester. The establishment of the conformations of the docked substrates was guided by the structure of the papain-leupeptin complex (1pop.pdb [24]). The geometries of the intermediates were optimized by using cycles of SD and adopted basis

Figure 2 For legend, see facing page

Figure 2 Examples of SF traces and associated residuals for the formation (a, c, e, g, i, k, m and o) and subsequent deacylation (b, d, f, h, j, l, n and p) of the dithioacyl-enzyme intermediates (2) in the hydrolysis of *N*-acetyl-Phe-Gly methyl thionoester (compound 1) catalysed by papain (a–h) and actinidin (i–p) at 25 °C, *I* 0.1, in aq. 4.2% (v/v) DMSO

For the papain reactions: at pH 3.25 (a–d), $[S]_0 = 77.5 \mu\text{M}$ and $[E]_T = 6.4 \mu\text{M}$; at pH 5.25 (e–h), $[S]_0 = 64.6 \mu\text{M}$ and $[E]_T = 4.6 \mu\text{M}$. For the actinidin reactions: at pH 3.70 (i–l), $[S]_0 = 152.2 \mu\text{M}$ and $[E]_T = 6.9 \mu\text{M}$; at pH 8.43 (m–p), $[S]_0 = 100.8 \mu\text{M}$ and $[E]_T = 7.8 \mu\text{M}$. (a), (b), (e), (f), (i), (j), (m) and (n) show the fits of the SF kinetic data (noisy signals) to single exponentials (smooth curves) and (c), (d), (g), (h), (k), (l), (o) and (p) are the associated residuals.

Newton–Raphson (ABNR) minimizations [20]. For the initial docking computations, the electrostatic interactions were screened using a distance-dependent dielectric function with an attenuation factor of 4. After the initial geometry optimizations, the actinidin-substrate system was solvated with a shell of water molecules 6 Å in thickness. The structures were again minimized using cycles of SD and ABNR.

Electrostatic-potential calculations

The models of the two enzymes and their derived dithioacyl-enzyme intermediates were established as described above. The electrostatic potentials at $N^{\delta 1}$ of the catalytic-site histidine Im side chains were calculated by solving the linearized Poisson–Boltzmann equation as described in [3,23], making use of the techniques reported in [20–22]. The choice of the value of the protein dielectric (7.5 for papain and 8.0–8.25 for actinidin) is discussed in the Results and discussion section. The charges on the catalytic-

site cysteinyl sidechains were partitioned at -0.8 on S^{γ} and -0.2 on C^{β} for the free enzymes and -0.19 on S^{γ} and $+0.19$ on C^{β} for the corresponding dithioacyl-enzymes (structures **2a** and **2b**). The difference between the two potentials ($\Delta\Delta G$) for a given enzyme divided by 1.3645 [23] provides the downward pK_a shift consequent on forming the dithioacyl-enzyme intermediate from the free enzyme.

The pK_a values of Asp¹⁶¹ of actinidin in both dithioacyl-enzyme and free-enzyme states were calculated by using the same computational techniques as outlined below with selected numbers of carboxy groups, including always the carboxy group of Asp¹³⁸, ionized as discussed in the Results and discussion section. The starting point was the pK_a value of Asp¹⁵⁸–CO₂H in papain [the carboxy group analogous to Asp¹⁶¹–CO₂H of actinidin] determined experimentally as 2.8 by using 4-chloro-7-nitrobenzofurazan (Nbf–Cl) as a reactivity probe [4]. This was used to calculate, in sequence, the pK_a values of Asp¹⁵⁸–CO₂H in the dithioacylpapain, Asp¹⁶¹–CO₂H in the dithioacylactinidin and finally this carboxy group in the free actinidin molecule.

RESULTS AND DISCUSSION

General characteristics of the pre-steady state (acylation) and steady-state (deacylation) phases of the hydrolysis of the methyl thionoester substrate (1) catalysed by papain and actinidin

The general form of the time courses for the hydrolysis of substrate **1** catalysed by the two cysteine proteinase variants with $[S]_0 \gg [E]_T$ at all pH values in the range ≈ 3.5 – 8.5 is that reported previously [5] for the hydrolysis of this substrate at pH 5.22. Thus, as expected, rapid formation of the dithioacyl-enzyme (ES'), detected by increase in A_{315} , is followed by slower loss of absorbance at this wavelength resulting from its hydrolysis (deacylation), which regenerates the catalytic site $Cys-S^-/His-Im^+H$ ion-pair. Examples of the results of the analysis of these pre-steady-state and steady-state phases of the time courses at low and high pH are shown in Figure 2.

The general form of the time courses is in accord with the minimal model of eqn (2), when the formation of the first transient (the adsorptive complex, ES) is complete within the deadtime of the SF system and the formation of the second transient (ES') is slow enough to be observed on the shorter of the two SF timescales. Exponential increase of $[ES']$ detected by increase in A_{315} to its steady-state value is followed by the rate-determining deacylation phase over a longer SF time scale. The latter eventually involves exponential decrease in A_{315} , which, at a given pH value, is independent of $[S]_0$ and is characterized by a first-order rate constant k , which is k_{+3} of both eqns (1) and (2) (see below). The results in Figure 2 illustrate the adherence to first-order kinetics of both the pre-steady-state (Figures 2a, 2e, 2i and 2m) and steady-state (Figures 2b, 2f, 2j and 2n) phases of catalysis at various pH values.

The steady state (deacylation)

The first-order decreases in A_{315} (such as those shown in Figures 2b and 2f for papain and Figures 2j and 2n for actinidin) provide values of the first-order rate constant (k). In terms of the minimal model of eqn (2), values of k are those of the rate constant for the hydrolysis of ES' (k_{+3}). No evidence was found either in [5] or in the present more extensive study that k might not characterize the hydrolysis of ES' catalysed by papain. For the reactions of actinidin, however, the kinetic data discussed below suggest that k might be characteristic, not of the hydrolysis of ES' , but rather, of a rate-determining conformational change in the $ES' \cdot P_1$ complex prior to deacylation as indicated in eqn (1).

The pre-steady state (acylation)

In terms of the model of eqn (2), the observed first-order rate constant, k_{obs} , for the pre-steady-state phase when the reaction is carried out with $[S]_0 \gg [E]_T$ (e.g. Figures 2a, 2e, 2i and 2m) is predicted to vary with $[S]_0$ according to eqn (3) [6]:

$$k_{obs} = \frac{k_{+2}[S]_0}{K_{m(acyl)} + [S]_0} + k_{+3} \quad (3)$$

in which $K_{m(acyl)} = (k_{-1} + k_{+2})/k_{+1}$, which approximates to $k_{-1}/k_{+1} = K_s$ when quasi-equilibrium around ES obtain ($k_{+2} \ll k_{-1}$, which is a necessary consequence of $k_{cat}/K_m \ll k_{+1}$ [25]).

Eqn (3) predicts that a plot of k_{obs} versus $[S]_0$ will contain a hyperbolic component with $k_{obs} = k_{+3}$ when $[S]_0 = 0$; that is, an ordinate intercept. Another way to show this is to plot $(k_{obs} - k_{+3})$ versus $[S]_0$, which has the effect of shifting the curve to go through

Figure 3 Demonstration of the linear dependence of $k_{obs} - k_{+3}$ on substrate concentration with a substantial ordinate intercept for the hydrolysis of *N*-acetyl-Phe-Gly methyl thionoester (compound **1**) catalysed by actinidin at pH 7.92

Other reaction conditions were as described in Figure 2. The experimental points are mean values for three determinations and the S.D. values were less than 10% of the means; k_{obs} is the observed first-order rate constant for the pre-steady-state phase of the catalysis and k_{+3} the first-order rate constant for the steady-state phase ($0.17 s^{-1}$), which is independent of substrate concentration at a given pH value. The continuous line is theoretical for eqn (8) and the following parameter values: k_{-2} (the ordinate intercept) $= 2.62 \pm 0.05 s^{-1}$ and $k_{+2}/K_{m(acyl)} = (1.53 \pm 0.02) \times 10^4 M^{-1} \cdot s^{-1}$.

0, 0; that is, $(k_{obs} - k_{+3}) = 0$ when $[S]_0 = 0$. If the latter plot does not go through 0, 0, or, in other words, the plot of k_{obs} versus $[S]_0$ has an ordinate intercept greater than the value of k_{+3} , then an additional term is required on the right-hand side of eqn (3); that is, k_{-2} as in eqn (7). Analysis of the conventional hyperbolic curves with $k_{obs} - k_{+3} = 0$ at $[S]_0 = 0$ (not shown) provided values of $K_{m(acyl)}$, k_{+2} and $k_{+2}/K_{m(acyl)}$ at the various pH values. These parameters and k_{+3} are related to the steady-state parameters by eqns (4) and (5), and their pH-dependence characteristics are presented and discussed below:

$$k_{cat} = \frac{k_{+2}k_{+3}}{k_{+2} + k_{+3}} \quad (4)$$

$$K_m = K_{m(acyl)} \cdot \frac{k_{+3}}{k_{+2} + k_{+3}} \quad (5)$$

In marked contrast with the conventional hyperbolic saturation curves found for papain, the plots of $k_{obs} - k_{+3}$ versus $[S]_0$ for actinidin are linear, importantly with non-zero ordinate intercepts (exemplified in Figure 3) at all of the pH values. Eqn (3) predicts linear plots when $[S]_0 \ll K_{m(acyl)}$, but the existence of ordinate intercepts requires an extended model, the simplest of which is eqn (6), in which the reversibility of ES' formation is acknowledged.

For this model $k_{\text{obs}} - k_{+3}$ is given by eqn (7) [26], which becomes eqn (8) when $[S]_0 \ll K_{\text{m(acyl)}}$:

$$k_{\text{obs}} - k_{+3} = \frac{k_{+2}[S]_0}{K_{\text{m(acyl)}} + [S]_0} + k_{-2} \quad (7)$$

$$k_{\text{obs}} - k_{+3} = \frac{k_{+2}}{K_{\text{m(acyl)}}} \cdot [S]_0 + k_{-2} \quad (8)$$

Eqn (8) shows that the linear plot of $k_{\text{obs}} - k_{+3}$ versus $[S]_0$ provides values of $k_{+2}/K_{\text{m(acyl)}}$ as the slope and k_{-2} as the ordinate intercept. The conceptual problem with the model of eqn (6) is the implausible assumption of significant conversion of ES' back to ES by P_1 in the low concentrations produced, if P_1 has diffused away from the enzyme. This problem is obviated in the hypothesis that an $\text{ES}' \cdot \text{P}$ complex has a lifetime long enough to permit intracomplex reaction of ES' with P_1 to re-form ES . A necessary aspect of this hypothesis is a slow conformational change to permit rapid desorption of the alcohol, P_1 , and its replacement by the water molecule required to hydrogen bond to His¹⁶²-Im (actinidin numbering) as part of the deacylation process. The simplest model incorporating this slow conformational change is eqn (1), in which $(\text{ES}' \cdot \text{P}_1)^*$ represents the conformation of the acyl-enzyme-alcohol complex from which the alcohol diffuses rapidly to leave $(\text{ES}')^*$, which binds water and undergoes deacylation. In terms of eqn (1), the pre-steady-state increase in A_{315} monitors the rapid, reversible formation of $\text{ES}' \cdot \text{P}_1$. The subsequent steady-state decrease in A_{315} monitors the hydrolysis of $(\text{ES}')^*$, preceded by the change in conformation from that of $\text{ES}' \cdot \text{P}_1$ to that of $(\text{ES}' \cdot \text{P}_1)^*$. It may be that following the release of P_1 from $(\text{ES}' \cdot \text{P}_1)^*$, the conformation of the acyl-enzyme returns to that of ES' prior to hydrolysis.

Investigation of the above hypothesis by pH-dependent kinetic study of the deacylation process in the reactions catalysed by actinidin and papain respectively

It was considered that study of the pH-dependence of k_{+3} would test the hypothesis that catalysis by actinidin requires a model like eqn (1), whereas catalysis by papain can be described by eqn (2). Thus, in eqn (2), k_{+3} characterizes the reaction of the dithioacyl-enzyme intermediate with water assisted by general base catalysis provided by the unprotonated imidazole group of His¹⁵⁹ (see [1,2] for reviews of the established features of the catalytic mechanism of papain). General base catalysis would be expected to be an absolute requirement, and this mechanism, therefore, predicts a plateau (pH-independent) value at high pH ($\bar{k}_{+3}$) which, in its simplest form, decreases with decrease in pH across a single sigmoid curve with a pK_a value associated with His¹⁵⁹-Im⁺H in the acyl-enzyme intermediate to approach zero at low pH.

Figure 4(a) suggests that the pH- k_{+3} profile for loss of the spectroscopic signal of the dithioacyl-papain intermediate **2a** does indeed conform to the type of sigmoidal dependence described above with $\bar{k}_{+3} = 0.825 \text{ s}^{-1}$ and pK_a 3.35. The simplest interpretation of the pK_a of 3.35 is that it is associated with protonic dissociation from His¹⁵⁹-Im⁺H. Lowe [27] reported pK_a values for the deacylation of several acyl-papains ranging from 3.5 to 3.85 and concluded, not unexpectedly, that the nature of the acyl group influences the pH-dependence of deacylation. In considering the observed pK_a value of 3.35 found in the present work, one factor that may need to be taken into account is the possible influence of Asp¹⁵⁸ [1], which could provide a value for the pK_a of His¹⁵⁹-Im⁺H somewhat greater than 3.35. The pK_a value of Asp¹⁵⁸-CO₂H in the free papain molecule has been determined to be 2.8 [4]. Thus

Figure 4 pH-dependence of the first-order rate constant (k_{+3}) for the steady-state loss of the dithioacyl-enzyme chromophore (k_{+3}) during catalysis of the hydrolysis of *N*-acetyl-Phe-Gly methyl thionoester (compound 1) by papain (a) and actinidin (b) at 25 °C and / 0.1

(a) The same continuous line is theoretical for both the pH-dependent rate equation for one reactive protonic state and a second, unreactive, state at low pH, $k_{+3} = \bar{k}_{+3}/(1 + [\text{H}^+]/K_1)$ and the following values of the characterizing parameters: $\text{pK}_a = 3.35 \pm 0.04$ and $\bar{k}_{+3} = 0.825 \pm 0.025 \text{ s}^{-1}$ and the pH-dependent rate equation for two reactive protonic states and a third, unreactive, state at low pH, $k_{+3} = \bar{k}_{+3}/(1 + [\text{H}^+]/K_1 + K_{II}/[\text{H}^+]) + \bar{k}'_{+3}/(1 + [\text{H}^+]^2/K_1K_{II} + [\text{H}^+]/K_{II})$ and the following values of the characterizing parameters: $\text{pK}_I = 2.80 \pm 0.04$, $\text{pK}_{II} = 3.60 \pm 0.05$, $\bar{k}_{+3} = 0.275 \pm 0.02 \text{ s}^{-1}$ and $\bar{k}'_{+3} = 0.825 \pm 0.03 \text{ s}^{-1}$. (b) The continuous line is theoretical for the pH-dependent rate equation for one kinetically influential ionization bounded by two reactive protonic states, $k_{+3} = \bar{k}_{+3}/(1 + [\text{H}^+]/K_1)$ and the following values of the characterizing parameters: $\text{pK}_I = 4.90 \pm 0.06$, $k_{+3} = 0.096 \text{ s}^{-1}$ (by inspection) and $\bar{k}'_{+3} = 0.17 \pm 0.01 \text{ s}^{-1}$.

if the same value is assumed for Asp¹⁵⁸-CO₂H in the acyl-enzyme intermediate **2a**, the data of Figure 4(a) fit equally well to two kinetically influential ionizations with $\text{pK}_I = 2.8$,

Figure 5 pH-dependence of the pre-steady state kinetic parameters of the catalysis of the hydrolysis of *N*-acetyl-Phe-Gly methyl thionoester (compound 1) by papain (a and b) and actinidin (c and d) at 25 °C and / 0.1

(a) The continuous line is theoretical for a pH-dependent rate equation for one reactive protonic state and a second, unreactive, state at low pH analogous to that shown in the legend to Figure 4(a) and the following values of the characterizing parameters: $pK_1 = 3.65$ and $\bar{k}_{+2}/\bar{K}_{m(acyl)} = 6.8 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ by curve-sketching; the broken line is theoretical for a pH-dependent rate equation for two reactive protonic states and a third, unreactive, state at low pH analogous to that shown in the legend to Figure 4(a) and the following values of the characterizing parameters: $pK_1 = 3.24$, $pK_{II} = 4.09$ both by analogy with values obtained independently using reactivity probes [2,3] $\bar{k}_{+2}/\bar{K}_{m(acyl)} = 3.2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $\bar{k}'_{+2}/\bar{K}'_{m(acyl)} = 6.9 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ both by curve-sketching using the stated pK_a values. (b) The continuous line is analogous to the broken line in (a) and the following values of the characterizing parameters: $pK_1 = 3.35$ and $\bar{k}_{+2} = 9.0 \text{ s}^{-1}$; $pK_{II} = 4.5$, $\bar{k}'_{+2} = 138 \text{ s}^{-1}$. (c) The continuous line is theoretical for a pH-dependent rate equation for two reactive protonic states and a third, unreactive, state at low pH analogous to that shown in the legend to Figure 4(a) and the following values of the characterizing parameters: $pK_1 = 2.70$, the pK_a value for ion-pair formation 2.70 ± 0.02 [28], $pK_{II} = 5.00$, the pK_a value obtained by analysis of the data in Figure 6, 5.00 ± 0.10 , $\bar{k}_{+2}/\bar{K}_{m(acyl)} = 8.40 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $\bar{k}'_{+2}/\bar{K}'_{m(acyl)} = 1.56 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ both by curve-sketching using the stated pK_a values. (d) The continuous line is theoretical for a pH-dependent rate equation for one kinetically influential ionization bounded by two reactive protonic states analogous to that shown in the legend to Figure 4(b) and the following values of the characterizing parameters: $pK_1 = 4.70 \pm 0.15$, $\bar{k}_{-2} = 2.00 \text{ s}^{-1}$ (by inspection) and $\bar{k}'_{-2} = 2.75 \pm 0.13 \text{ s}^{-1}$.

$\bar{k}_{+3} = 0.275 \text{ s}^{-1}$ and $pK_{II} = 3.6$, $\bar{k}'_{+3} = 0.825 \text{ s}^{-1}$ (Figure 4a), the latter being associated with His¹⁵⁹-Im⁺H.

It is a striking observation that, by contrast with the pH- k_{+3} profile for the papain reaction (Figure 4a), the shape of the profile for the analogous actinidin reaction (Figure 4b) is quite different. This pH- k_{+3} profile is a shallow sigmoid in which the value of

k_{+3} is only little affected by the state of ionization of a low pK_a acid, with substantial reactivity in both of the ionization states that bound the pK_a of 4.9 ($\bar{k}_{+3} = 0.096 \text{ s}^{-1}$ and $\bar{k}'_{+3} = 0.17 \text{ s}^{-1}$). This form of pH-dependence is not that which would be expected for an obligatory general-base-catalysed hydrolysis reaction. This result, therefore, supports the hypothesis that for the actinidin

reaction k_{+3} reflects a process other than hydrolysis of the dithioacylactinidin (**2b**). In this interpretation the kinetically influential ionization with pK_a 4.9 exerts a small effect on the postulated rate-determining conformational transition $[ES' \cdot P_1 \rightarrow (ES' \cdot P_1)^*]$ of eqn 1] suggested to be required to permit the rapid replacement of P_1 by water and the subsequent hydrolysis step $[(ES' \cdot P_1)^* \rightarrow P_1 + (ES')^* \rightarrow E + P_2]$. The possibility that the pK_a of 4.9 might be one of the electrostatic modulators with a similar pK_a value (4.85–5.2) in the free enzyme that affects acylation and the retrogression of $ES' \cdot P_1$ to ES is discussed below.

pH-dependent kinetic study of the acylation process

For the papain reaction, the pH – $k_{+2}/K_{m(acyl)}$ profile (Figure 5a) is, as expected, a conventional sigmoidal curve with the reactivity increasing with increase in pH . The data fit well to a single kinetically influential pK_a of 3.65 [$\tilde{k}_{+2}/\tilde{k}_{m(acyl)} = 6.8 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$], but this is not a previously observed free enzyme pK_a value for papain. It is known from earlier studies [2,3], however, that Cys²⁵–His¹⁵⁹ ion-pair formation in papain (pK_a 3.35) is not sufficient for full catalytic competence, which requires also deprotonation of an electrostatic modulator across pK_a 4.0. It was not unexpected, therefore, that the data of Figure 5(a) fit well also to a curve with observed pK_a values of 3.24 and 4.09, values shifted from 3.35 and 4.0 respectively by their proximity [3]. The corresponding pH – k_{+2} profile (Figure 5b) is of similar form to that of Figure 5(a), but with the major kinetically influential pK_a value of 4.5 (with $\tilde{k}_{+2} = 138 \text{ s}^{-1}$), with a minor contribution from ion-pair formation (pK_a 3.35, $\tilde{k}_{+2} = 9.0 \text{ s}^{-1}$). The simplest interpretation of this is that the electrostatic modulator with pK_a 4.0 in the papain molecule is perturbed to pK_a 4.5 in the ES complex.

For the actinidin reaction the pH – $k_{+2}/K_{m(acyl)}$ profile in the pH region ≈ 4 –8.5 (Figure 5c) is probably a double sigmoidal curve with the reactivity increasing with increase in pH . The data fit well to pK_a 2.7, the value for ion-pair formation in the free actinidin molecule ($\tilde{k}_{+2}/\tilde{k}_{m(acyl)} = 8.4 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$) and pK_a 5.0 ($\tilde{k}_{+2}/\tilde{k}'_{m(acyl)} = 1.56 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$). As predicted, the (more extensive) (k_{cat}/K_m) – pH data for the hydrolysis of *N*^α-benzoyl-L-arginine 4-nitroanilide catalysed by actinidin in acidic media (Figure 6) also fit well to these same two kinetically influential pK_a values. Use of a new, highly reactive, thiol-specific, substrate-derived reactivity probe, (2-(*N*-acetylphenylalanyl-amino)ethyl 3-pyridazinyl disulphide, defined the kinetically significant pK_a values in the free actinidin molecule particularly clearly as 2.7 CCys²⁵/His¹⁶² ion-pair formation), 4.9 and 6.7 (electrostatic modulators) and 9.6 (ion-pair deprotonation) [28]. The pK_a of 5.0 in the profile of Figure 5(c) is presumably associated with the electrostatic modulator (pK_a 4.9) detected by thiol-specific reactivity probes [28–31], and might be the same modulator (pK_a 4.9) discussed above in connection with the pH -dependence of k_{+3} (see below). Evidence that the pK_a of this modulator might be only slightly perturbed when the enzyme is in complex with, or derivatized by, the substrate is provided by the pH -dependence of k_{-2} (the retrogression of $ES' \cdot P_1$ to ES in eqn (1) (Figure 5d). This shallow sigmoidal curve is characterized by a pK_a value of 4.7, bounded by pH -independent values of k_{-2} of 2.0 s^{-1} and 2.75 s^{-1} .

Investigation of the kinetically influential ionization with $pK_a \approx 5$ in actinidin by computer modelling

The question of the possible identity of the ionizing group with pK_a 4.9 that modulates the k_{+3} step in the actinidin-catalysed reaction (Figure 4b) was investigated by computer modelling.

Figure 6 pH -dependence of k_{cat}/K_m for the catalysis of the hydrolysis of *N*^α-benzoyl-L-arginine 4-nitroanilide by actinidin in 8% (v/v) *N,N*-dimethylformamide at 25 °C, / 0.1

The points are experimental and the continuous line is theoretical for a pH -dependent rate equation for two catalytically active states and the following values of the parameters: $pK_1 = 2.70$, the pK_a value for ion-pair formation 2.70 ± 0.02 [28], $pK_{II} = 5.00 \pm 0.10$, $pK_{III} = 9.50 \pm 0.20$, $\tilde{k}_{cat}/K_m = 2.80 \text{ M}^{-1} \cdot \text{s}^{-1}$ (by inspection and curve-sketching) and $\tilde{k}'_{cat}/\tilde{K}'_m = 13.50 \pm 1.20 \text{ M}^{-1} \cdot \text{s}^{-1}$.

The two most obvious alternatives are (i) His¹⁶²–Im⁺H in the dithio-acylenzyme intermediate (**2b**) and (ii) the unknown group with $pK_a \approx 5$ that modulates the acylation process in catalysis (Figures 5c and [6]), the reactions of the Cys²⁵–S[−]/His¹⁶²–Im⁺H ion-pair with thiol-specific reactivity probes [28–31] and the retrogression of $ES' \cdot P_1$ to ES in eqn (1) (Figure 5d). If it were the former, the role of His¹⁶² detected by the pH -dependence of k_{+3} would be additional to its subsequent (non-rate-determining) role in the catalytic process of general base catalysis of the hydrolytic step, for which the pH – k profile would be of form of Figure 4(a).

An attempt to examine these possibilities was made by use of electrostatic potential calculations. The approach initially was to calculate the protein dielectric of papain that provides the pK_a value of His¹⁵⁹–Im⁺H in the dithioacyl-papain (compound **2a**) observed experimentally and to use this as the basis on which to calculate the unknown pK_a value of His¹⁶²–Im⁺H in the analogous dithioacyl-actinidin (compound **2b**). The value obtained from Figure 4(a) is 3.35 or, as seems more likely, 3.6 if the observed pK_a value is a composite of those of His¹⁵⁹–Im⁺H and Asp¹⁵⁸–CO₂H, as discussed above. Solution of the Poisson–Boltzmann equation provides the lower value (pK_a 3.35) when a protein dielectric of 7.2 is used and the higher value (pK_a 3.6) when a protein dielectric of 7.5 is used. These values of protein dielectric are well within the range that has been found to characterize complex dielectric profiles of proteins [32]. When a dielectric of 7.5 is used also for actinidin, the calculated pK_a value of His¹⁶²–Im⁺H in the dithioacylactinidin is 5.4. To ascertain whether a dielectric of 7.5 is appropriate for the actinidin derivative, each of the dithioacyl-enzymes were subjected to MD simulations at 300 K for 200 ps using the conditions described in [5]. The root-mean-square fluctuations of the dipole moments due to the dipoles within 10 Å of the Cys–His ion-pairs were found to be 7–10% larger in the actinidin derivative. This difference (see [33]) leads to a slightly

Figure 7 Views of the catalytic site regions of (a) papain, (b) caricain and (c) actinidin showing the hydrogen-bonding interactions with the side chains of Asp¹⁵⁸ (a and b) and Asp¹⁶¹ (c) and their disposition with respect to the catalytic sites (Cys²⁵ and His¹⁵⁹ in a and b, and Cys²⁵ and His¹⁶² in c)

(a) Three hydrogen bonds are donated to Asp¹⁵⁸–CO₂[−] in papain by Ala¹³⁶ and Ala¹³⁷; these are predicted to account for the low pK_a of Asp¹⁵⁸–CO₂H (2.8). (b) Five hydrogen bonds are donated to Asp¹⁵⁸–CO₂[−] in caricain by Ser¹³⁶ and Lys¹³⁷; these, which include one by the cationic side chain of Lys¹³⁷ are predicted to account for the lower pK_a value of Asp¹⁵⁸–CO₂H (2.0). (c) In actinidin O^{δ2} of both Asp¹³⁸ and Asp¹⁶¹ can share a proton when one of these side chains is anionic. As a result one of these carboxy groups is predicted to have a low pK_a and one a high pK_a. For further details, see the text.

larger (by < 10 %) value of the dielectric constant of the active-centre region of actinidin relative to that of papain. If the upper limit (7.5 + 0.75 = 8.25) is taken as the dielectric of actinidin, the pK_a of His¹⁶²–Im⁺H in its dithioacyl derivative is calculated to be 5.7 (5.6 if the dielectric is taken to be 8.0). This value is greater than the value (4.9) found experimentally (Figure 4b) to modulate the *k*₊₃ step (see below for discussion of this discrepancy following the report of the investigation of Asp¹⁶¹–CO₂H as a potential source of modulation).

We next turned our attention to Asp¹⁶¹–CO₂H. The modulators in papain and caricain with the lowest experimentally observed pK_a values (≈ 4; [3]) cannot be assigned to Asp¹⁵⁸–CO₂H, the potential source of electrostatic modulation closest to the common Cys–S[−]/His–Im⁺H catalytic-site ion pair. This is because the pK_a values of Asp¹⁵⁸–CO₂H in papain and caricain have been shown to be much less than 4 (2.8 in papain and 2.0 in caricain [4]). The former was determined experimentally by using Nbf–Cl as a reactivity probe and the latter by Poisson–Boltzmann calculations. The low pK_a values of the Asp¹⁵⁸ carboxy groups can be accounted for by the hydrogen-bonding interactions shown in Figures 7(a) and 7(b). The five hydrogen bonds donated to Asp¹⁵⁸–CO₂[−] in caricain (by Ser¹³⁶ and Lys¹³⁷) as against the three in papain (by Ala¹³⁶ and Ala¹³⁷) might be expected to produce greater stabilization of the Asp¹⁵⁸–CO₂[−] anion in the case of caricain and hence a lower pK_a value. This low pK_a in caricain explains why the high reactivity towards Nbf–Cl at low pH observed with papain, which depends on donation of a hydrogen bond from the undissociated Asp¹⁵⁸–CO₂H to Nbf–Cl, is not observed with caricain [4]. Analogous lack of reactivity at low pH observed with actinidin [34], however, seems unlikely to be explained by a low pK_a value for Asp¹⁶¹–CO₂H. Unlike

Scheme 1 Perturbation of pK_a values in model dicarboxylic acid systems

Perturbation in maleic acid (a) derives from intramolecular hydrogen bonding in the central monoionic state which is not possible in the *trans* isomer fumaric acid (b).

the situation in caricain, there are no cationic neighbours to stabilize the carboxylate anion. Rather, uniquely in actinidin, an important influence on Asp¹⁶¹ could be the neighbouring side chain of Asp¹³⁸ (Figure 7c). This potential influence has been commented on previously (see [35] and references cited therein). The O^{δ2} side-chain oxygen atoms of Asp¹³⁸ and Asp¹⁶¹ are separated by only 3.9 Å and may share a proton when one side chain (depicted in the discussion that follows to be that of Asp¹³⁸) is anionic. Low-*M_r* dicarboxylic acid models constrained to provide intramolecular hydrogen bonding in the monoionic form demonstrate the predicted perturbation of the two pK_a values. Thus the first and second pK_a values of maleic acid (Scheme 1a)

are perturbed to lower and higher values respectively relative to those of the *trans* isomer, fumaric acid (Scheme 1b), where the analogous stabilization of the monoionic form is not possible (see [36]). Thus, by contrast with the situations in papain and actinidin, where the pK_a values of Asp¹⁵⁸-CO₂H are abnormally low, the unique environment of Asp¹⁶¹-CO₂H in actinidin could result in an abnormally high pK_a value. This could make the Asp¹³⁸/Asp¹⁶¹ couple a candidate for the modulator with $pK_a \approx 5$. It is the non-protonated O³¹ atom of Asp¹⁶¹ which faces the catalytic site of actinidin and is closest to the Nbf-Cl ligand. This would explain the lack of enhancement of reactivity at low pH.

Poisson-Boltzmann calculations starting from the experimentally determined pK_a value of Asp¹⁵⁸-CO₂H in papain (2.8) provided a pK_a value of Asp¹⁶¹-CO₂H in the dithioacyl-actinidin in the range 4.5–5.7 and a value in the free actinidin molecule of 4.8–6.0, the value in each case increasing with increase in the number of aspartic acid and glutamic acid side chains in addition to that of Asp¹³⁸ assumed to be ionized. The calculated pK_a values of both Asp¹⁶¹-CO₂H and His¹⁶²-Im⁺H are similar to each other and somewhat higher than the kinetically influential pK_a values observed. Electrostatic potential calculations, however, are known to be sensitive to co-ordinate errors and charge distributions [37] and the calculated and experimental values could be considered to correspond reasonably well within the anticipated errors. The calculations certainly demonstrate a high second pK_a value of the Asp¹³⁸-CO₂H/Asp¹⁶¹-CO₂H couple which was predicted from structural considerations.

As far as the modulator of k_{+3} (Figure 4b) is concerned, the similarity in the calculated pK_a values of Asp¹⁶¹-CO₂H and His¹⁶²-Im⁺H does not permit a choice between these candidates on this basis alone. Structural considerations of the cavity predicted to undergo the rate-determining conformational change, however, suggest that Asp¹⁶¹ might be the preferred candidate. Thus the Asp¹⁶¹-CO₂⁻ anion forms hydrogen bonds with the backbone of Ala¹³⁹ and possibly also with Ala¹⁴⁰. Both of these residues act as a hinge for movements of the contiguous loop that includes the Asp¹⁴²-Lys¹⁴⁵ salt bridge and forms the cavity. Any perturbation of Asp¹⁶¹ such as ionization would be expected to affect the dynamics of this loop region. By contrast, His¹⁶² is tethered by a hydrogen bond to Asn¹⁸² and constrained by the hydrophobic environment provided by Trp¹⁸⁴ and does not make direct contacts with the loop region. A change in the ionization state of His¹⁶²-Im⁺H, therefore, seems less likely to affect its dynamics. By contrast with the uncertainty about the identity of the modulator of k_{+3} , His¹⁶² can be ruled out as the modulator with $pK_a \approx 5$ of $k_{+2}/K_{m(\text{acyl})}$ (Figure 5c), the identical quantity k_{cat}/K_m (Figure 6) and k for reactions of time-dependent inhibitors [18–31] because the pK_a of His¹⁶²-Im⁺H of the free actinidin molecule is 9.5–9.6 (Figure 6 and [28–31]).

Concluding comment

The different types of behaviour exhibited by papain and actinidin over the pH range ≈ 3.5 –8.5 provide evidence for a post-acylation conformational change in the case of the actinidin dithioacyl-enzyme intermediate. In particular, only in the case of papain is the pH- k_{+3} profile (a conventional sigmoidal curve that approaches $k_{+3} = 0$ at low pH) consistent with rate-determining, obligatory, general-base-catalysed reaction of a water molecule with the dithioacyl-enzyme intermediate. The analogous profile in the case of actinidin (a shallow sigmoid bounded by two states of similar, but not identical, reactivity) is not consistent with such an interpretation, but supports the hypothesis that k_{+3} characterizes the postulated conformation change, modulated electrostatically

by a group with $pK_a \approx 5$. Electrostatic modulation by a group with this pK_a value is observed also in the acylation process, the reaction of the Cys²⁵-S⁻ with time-dependent inhibitors, and the retrogression of ES'·P₁ to ES. The case is made for the assignment of the Asp¹³⁸/Asp¹⁶¹ couple as the source of electrostatic modulation of various reactions of the actinidin catalytic site with $pK_a \approx 5$. This would be analogous to electrostatic modulation by Asp¹⁵⁸ in both papain and caricain. The difference, however, is that the low pK_a values of Asp¹⁵⁸-CO₂H means that this modulator is essentially fully ionized in the pH range where catalytic activity is detected and cannot be the kinetically observed modulator with $pK_a \approx 4$. Modulation by Asp¹⁵⁸ in these two enzymes is demonstrated by the much decreased catalytic activities of the Asp¹⁵⁸ → Asn mutants [38,39] and may be regarded, therefore, as being in the permanently 'on' state.

The conclusion relating to k_{+3} is a significant contribution to the accumulating evidence that papain and actinidin lie at the extremes of the spectrum of active-centre behaviour exhibited by several members of the papain family [1]. This spectrum of behaviour, hitherto detected by kinetic studies using thiol-specific substrate-derived reactivity probes (e.g. [28–31]) and relating to the acylation process, has now been shown to apply also to post-acylation events. All of these differences appear to depend on variation in changes in protein geometry promoted by electrostatic effects and, in some cases at least, also by binding interactions. The modulatory role for the carboxylate anion closest to the catalytic site Cys-S⁻/His-Im⁺H is now more firmly established as a generality for those enzyme variants where it exists. The difference in this respect between papain and caricain on the one hand and actinidin on the other is that, in the former enzymes, Asp¹⁵⁸ is permanently 'switched on' whereas in actinidin the activation by Asp¹⁶¹-CO₂H is demonstrable by pH-dependent kinetic studies. Even for variants in which there is no exact analogue of Asp¹⁵⁸/Asp¹⁶¹ such as cathepsins B (Gly¹⁹⁶) and H (Asn¹⁶⁵) (see [1]) and interleukin-1 β converting enzyme ('ICE'; see [3]), electrostatic modulation may well be provided by other components of the electrostatic fields. For example, Asp¹⁸⁵ has been suggested as a possible candidate for electrostatic modulation with pK_a 5.5 in interleukin-1 β converting enzyme [3].

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