Variation in aspects of cysteine proteinase catalytic mechanism deduced by spectroscopic observation of dithioester intermediates, kinetic analysis and molecular dynamics simulations

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The possibility of a slow post-acylation conformational change during catalysis by cysteine proteinases was investigated by using a new chromogenic substrate, *N*-acetyl-Phe-Gly methyl thionoester, four natural variants (papain, caricain, actinidin and ficin), and stopped-flow spectral analysis to monitor the pre-steady state formation of the dithioacylenzyme intermediates and their steady state hydrolysis. The predicted reversibility of acylation was demonstrated kinetically for actinidin and ficin, but not for papain or caricain. This difference between actinidin and papain was investigated by modelling using QUANTA and CHARMM. The weaker binding of hydrophobic substrates, including the new thionoester, by actinidin than by papain may not be due to the well-known difference in their S₂-subsites, whereby that of actinidin in the free enzyme is shorter due to the presence of Met²¹¹. Molecular dynamics simulation suggests that during substrate binding the sidechain of Met²¹¹ moves to allow

full access of a Phe sidechain to the S₂-subsite. The highly anionic surface of actinidin may contribute to the specificity difference between papain and actinidin. During subsequent molecular dynamics simulations the P₁ product, methanol, diffuses rapidly (over < 8 ps) out of papain and caricain but ‘lingers’ around the active centre of actinidin. Uniquely in actinidin, an Asp¹⁴²–Lys¹⁴⁵ salt bridge allows formation of a cavity which appears to constrain diffusion of the methanol away from the catalytic site. The cavity then undergoes large scale movements (over 4.8 Å) in a highly correlated manner, thus controlling the motions of the methanol molecule. The changes in this cavity that release the methanol might be those deduced kinetically.

Key words: actinidin, caricain, ficin, papain, rate-determining conformational change.

INTRODUCTION

The well-established features of the cysteine proteinase mechanism, reviewed in [1] and outlined below, have been demonstrated principally for papain (EC 3.4.22.2), the much studied minor cysteine proteinase component of the latex of *Carica papaya*. Some of these features have been demonstrated also for some other members of the papain family, notably caricain [papaya proteinase Ω (EC 3.4.22.30), a major companion cysteine proteinase component of papaya latex], actinidin (EC 3.4.22.14) from *Actinidia chinensis*, and ficin (EC 3.4.22.3) from *Ficus glabrata*. The minimal model of catalysis of the hydrolysis of an amide or ester substrate (S) by a cysteine proteinase (E) [equation (1)] involves simultaneous formation of an acylenzyme intermediate (ES′) and the amine or alcohol product (P₁) by reaction within an adsorptive complex (ES) followed by hydrolysis (deacylation) of ES′, which provides the carboxylate product (P₂):

Evidence for the existence of the adsorptive complex, ES, is provided by the multiphase stopped-flow kinetic records of reactions with peptide derivatives tagged with a fluorescent

reporter group [2], and the plausible indications of enzyme–substrate interactions deduced from model building on the basis of crystal structures of enzyme–inhibitor combinations [3]. The evidence for the acylenzyme intermediate, ES′, is strong and consists of a variety of kinetic and spectroscopic data (reviewed in [1]). Particularly convincing evidence that ES′ exists as a thioester intermediate was provided by its direct spectroscopic observation [4] in the hydrolysis of methyl thionohippurate (benzoylglycine methyl thionoester, **1**, Figure 1). This thionoester reacts with the catalytic site thiol group of papain (Cys²⁵) to produce the dithioacylenzyme (**2**, Figure 1) as an intermediate in the catalytic process with characteristic UV absorption (λ_{max} close to 315 nm). The dithioester intermediate differs from the predicted natural intermediate (**3**, Figure 1) by the substitution of C=S for the C=O group. The values of *k*_{cat}/*K*_m are similar for oxygen and thionoester substrates, however, and it seems probable that many conclusions drawn from experiments on thionoester substrates will apply also to the oxygen analogues. Subsequent use of thionoester substrates has hitherto concentrated on applying resonance Raman spectroscopy to characterize the conformations of the acyl groups in dithioacylenzyme intermediates (see e.g. [5,6]).

In the present work, we have synthesized a new thionoester substrate, *N*-acetylphenylalanylglycine methyl thionoester (*N*-

Abbreviations used: Boc, t-butyloxycarbonyl; MD, molecular dynamics; m.p., melting point; SF, stopped-flow; THI, tetrahedral intermediate; z, benzyloxycarbonyl.

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Figure 1 Substrates (1, 4–6) and acylenzyme intermediates (2, 3, 7) pertaining to cysteine proteinase mechanism and its kinetic investigation

acetyl-Phe-Gly methyl thionester, **4**, Figure 1) which contains the Phe residue at P_2 to provide proper occupancy of the S_2 -subsite. We then used it to investigate kinetically the catalytic pathway of four variants of the papain family: caricain, ficin, actinidin and papain itself. We have demonstrated previously [1,7,8], using other experimental approaches, the value of using variants of this enzyme family to reveal gradations in functional characteristics and mechanistic phenomena that are more easily demonstrated in some members than in others.

The formation of ES' undoubtedly proceeds through the formation of a tetrahedral species, but whether this involves a discrete tetrahedral intermediate (THI) or a concerted mechanism involving only a tetrahedral transition state is uncertain [1]. Regeneration of enzyme in the catalytic cycle occurs by hydrolysis of ES' catalysed by (His¹⁵⁹)-imidazole (papain numbering) as a general base. Proton-inventory analysis of the minimal model [9] suggested that the transition state for the deacylation of acyl papains is vibrationally well-coupled and lies on a concerted reaction pathway. This was supported subsequently by computational analysis [10].

Recent experiments using thiol-specific reactivity probes and *N*-acetyl-Phe-Gly-4-nitroanilide (**5**, Figure 1), a substrate containing the same recognition features as the thionoester substrate **4**, revealed facets of cysteine proteinase mechanism additional to those associated with equation (1), and the putative THIs between ES and ES' and between ES' and $E + P_2$ [7,8]. It had long been considered axiomatic that catalytic competence of cysteine proteinases mirrors the generation of the ubiquitous catalytic site

imidazolium–thiolate ion-pair, the motif that provides the nucleophilic and acid–base chemistry required to produce the acylenzyme intermediate ES' and to effect its subsequent hydrolysis. A combination of reactivity probe and catalysis kinetics supported by electrostatic potential calculations, however, showed that the ion-pair state is fully developed at low pH where the enzymes are essentially inactive [7]. Catalytic competence requires additional protonic dissociation with pK_a close to 4. We suggest that this allows modulation of ion-pair geometry from the 'intimate' ion-pair state in which the anionic and cationic components are maximally mutually solvated to one in which the thiolate and imidazolium components are positioned to play their roles in acylenzyme formation as nucleophile and general acid catalyst respectively. Although (Asp¹⁵⁸)-CO₂[−] is necessary for significant catalytic ability, this is not the electrostatic modulator produced by protonic dissociation with pK_a 4, whose origin remains to be discovered. Thus the pK_a value of (Asp¹⁵⁸)-CO₂H was determined to be 2.8 in papain by using 4-chloro-7-nitrobenzofurazan as a reactivity probe, and calculated to be 2.0 in caricain by solving the linearized Poisson–Boltzmann equation [8].

The electrostatic modulation of ion-pair geometry referred to above was deduced by using a combination of 4,4'-dipyrimidyl disulphide as a reactivity probe and *N*-acetyl-Phe-Gly-4-nitroanilide (**5**, Figure 1) as substrate [7]. Additional modulation, provided by specific binding interactions, was detected by using substrate-derived 2-pyridyl disulphide reactivity probes [11–15]. These results prompted the investigation, in the present work, of the possibility of conformational change in the catalytic process itself. The starting point was the interesting study on ficin catalysed hydrolysis reported by Hollaway and Hardman [16]. They used benzyloxycarbonyl (Z)-Lys-4-nitrophenylester (**6**, Figure 1) which contains an electronically activated, chromophoric leaving group. Release of 4-nitrophenol, monitored at 347 nm, was found to closely parallel proton release monitored at 576 nm using Chlorophenol Red as an indicator. These results were interpreted as simultaneous release of both P_1 and P_2 into bulk solution. This was postulated to involve a slow conformational change in the $ES' \cdot P_1$ complex followed by rapid deacylation of ES' to $E + P_2$ to complete the catalytic cycle.

In the present work we sought evidence for a relatively long-lived $ES' \cdot P_1$ complex, and the reversibility of its formation by direct observation of ES' using the new specific thionoester substrate, *N*-acetyl-Phe-Gly methyl thionester (**4**, Figure 1). This provides the opportunity to monitor the formation (and subsequent hydrolysis) of the dithioester moiety of the ES' (**7**, Figure 1) component of the postulated $ES' \cdot P_1$ complex by spectral analysis at 315 nm. By contrast with Z-Lys-4-nitrophenyl ester (**6**, Figure 1), **4** resembles more closely natural substrates in that it contains the recognition features required to provide a good fit in the S_2 -subsite and S_1 – S_2 intersubsite regions of papain and caricain and a small, non-aromatic, non-electronically-activated leaving group. These design features and electrical neutrality were incorporated to obviate potential complications from electrostatic effects involving the substrate, a 'sticky' aromatic leaving group, and the electronically-assisted leaving-group departure, and to provide for simple direct observation of ES' and the possible influence of key recognition features in the substrate.

The pre-steady state kinetics of the formation of the dithioester chromophore of ES' on the stopped-flow time scale with $[S]_0 \gg [E]_T$, established ES' formation for ficin and for actinidin to be reversible as required for the existence of the postulated relatively long-lived $ES' \cdot P_1$ complexes. These results, taken together with those of Hollaway and Hardman [16] for ficin, are considered in

terms of a five-step model in which the isomerization of $ES' \cdot P_1$ to a conformer that allows rapid release of P_1 into bulk solvent is rate determining. There is no convincing evidence for analogous reversibility of $ES' \cdot P_1$ formation for papain and caricain, where the binding of the substrate is much tighter. These differences, explored by computer modelling, and the variation in the rate constants observed for the steady-state loss of the dithioester chromophore of ES' for the four enzymes are discussed. Molecular dynamics (MD) simulations suggest that the low affinity of actinidin for hydrophobic substrates may not be due entirely, if at all, to a distorted binding mode in a truncated S_2 -subsite, but rather, in part at least, to the highly-charged surface of the enzyme. It is of particular interest that they demonstrate also that the methanol product (P_1) of the acylation reaction is retained within the catalytic-site region for longer in actinidin than in papain and caricain. This appears to be due to the existence, uniquely in actinidin, of the Asp¹⁴²–Lys¹⁴⁵ salt bridge which allows the formation of a cavity that constrains diffusion of the methanol molecule. Subsequent changes in this cavity leading to release of the methanol might be those deduced kinetically.

EXPERIMENTAL

Enzymes and active-centre titrant

Procedures for the purification of actinidin [17], caricain [14], ficin [18] and papain [19], which include those for the production of the fully-active enzymes by covalent chromatography, have been described previously, as has their evaluation by spectroscopic titration at 343 nm ($\epsilon_{343} = 8080 \text{ M}^{-1} \cdot \text{cm}^{-1}$) using 2,2'-dipyridyl disulphide as titrant [20]. 2,2'-Dipyridyl disulphide (Aldrich) was purified as described previously [17].

Synthesis of *N*-acetylphenylalanylglycine methyl thionoester (4)

Powdered aminoacetonitrile hydrochloride (Lancaster Synthesis, Easgate, Lancs., U.K.; 1.30 g, 14 mmol) was suspended by stirring in CH_2Cl_2 (100 ml), and deprotonated with di-isopropyl-ethylamine (2.4 ml, 14 mmol). After stirring for 60 min at room temperature (22 °C), the solution was cooled to –10 °C and *t*-butoxycarbonyl (Boc)-phenylalanine *N*-hydroxysuccinimide ester (Sigma; 5.07 g, 14 mmol) was added gradually, with stirring. The reaction mixture was allowed to reach room temperature during further stirring over 12 h. After washing sequentially with 1% (w/v) aqueous citric acid solution, saturated aqueous NaHCO_3 solution and saturated aqueous NaCl solution, the organic layer was dried with anhydrous Na_2SO_4 , and evaporated to dryness after removal of the drying agent by filtration. Crystallization of the resulting solid from *n*-propanol/water gave Boc-Phe-NHCH₂CN [3.75 g, 85% yield; melting point (m.p.), 138 °C].

The Boc derivative was dissolved in formic acid (10% w/v) and left to stand at room temperature for 12 h before removing the formic acid *in vacuo*. The residue was then dissolved in water (15 ml), and after addition of excess NaHCO_3 , acetic anhydride (1.5 mol equivalent) was added dropwise with vigorous stirring over 15 min. After stirring for 30 min at 20 °C, the acetylated product, (*N*-acetyl-Phe-NHCH₂CN) was precipitated by addition of water (50 ml), collected by filtration and washed with water. Crystallization from *n*-propanol/water gave a chromatographically homogeneous (TLC; ethyl acetate/methanol, 5:1, v/v) product with a m.p. of 190–192 °C.

This nitrile (1 g) was dissolved in dry CH_2Cl_2 (20 ml) containing dry methanol (0.5 ml) and cooled to 0 °C. Excess HCl gas was bubbled into the solution, which was then allowed to stand at

4 °C for 24 h. Excess dry diethylether was then added to precipitate the imidoester salt as a granular white solid. This was washed with dry diethylether by decantation and then dried over NaOH *in vacuo*. The imidoester product (1.2 g) was dissolved in ice-cold methanol/pyridine (1:5, v/v) and the solution was saturated with H_2S at 0 °C. The reaction flask was stoppered and allowed to reach room temperature, after which the solvents were removed *in vacuo*. The residue was partitioned between CH_2Cl_2 and 10% (w/v) aqueous citric acid after which the thionoester (4) was recovered from the CH_2Cl_2 phase by drying over anhydrous Na_2SO_4 , filtration and evaporation. Crystallization from ethyl acetate gave 0.4 g of pure material by TLC (silica gel; CH_2Cl_2 /ethyl acetate, 1:1). After recrystallization from ethyl acetate the thionoester product (4) had the following characteristics: m.p., 142–143 °C; $[\alpha]_D^{25} = -11.4^\circ$ (c 1.06, methanol); elemental analysis: C 57.81%, H 6.15%, N 9.68% ($\text{C}_{14}\text{H}_{19}\text{N}_2\text{O}_3\text{S}$ requires C 57.20%, H 6.17%, N 9.53%); mass spectrum, m/z 295.0 (M^+) ($\text{C}_{14}\text{H}_{19}\text{N}_2\text{O}_3\text{S}$ requires 295.4); $^1\text{H-NMR}$ (CD_3Cl): δ 2.0 (s, 3H, CH_3CO), δ 3.1 [m, 2H, $-(\text{CH}_2)-\text{Gly}$], δ 4.1 (s, 3H, $-\text{CH}_3$), δ 4.8 (m, H, $-\text{CH}-\text{Phe}$), δ 6.1 (m, H, $-\text{NH}-$), δ 6.6 (m, H, $-\text{NH}-$), 7.2 (m, 6H, Phe-ring).

Stopped-flow (SF) kinetics

All reactions were carried out at 25 °C and I 0.1 in solutions containing 1 mM EDTA and 4.2% (v/v) aqueous DMSO, 20–600 μM thionoester substrate and 1.2–4.65 μM enzyme such that $[\text{S}]_0 > 10[\text{E}]_0$. Solvents were degassed under reduced pressure prior to preparation of reactant solutions. Kinetic studies were performed with an SF.17MV SF spectrophotometer, kinetics workstation, data-acquisition and analysis software (Applied Photophysics, Leatherhead, Surrey, U.K.). Monochromator entrance and exit slits were set to 1 mm. Formation and subsequent hydrolysis of the dithioacylenzyme intermediates were monitored at 315 nm. A_{315} -versus-time results were recorded over 100 s for the reactions of papain and actinidin, and over 200 s for the reactions of caricain and ficin, in order to observe the rapid formation of the dithioacylenzyme intermediate and analyse its first-order decay to provide the first-order rate constant (k). The reactions were also monitored over 200 ms for papain, 1 s for actinidin and caricain, and 2 s for ficin, which isolates the increase in A_{315} in order to obtain the observed first-order rate constant (k_{obs}) for the formation of the dithioacylenzymes. The rate constants were evaluated by fitting the A_{315} -versus-time results, collected by the Acorn Archimedes microcomputer (Cambridge, Cambridgeshire, U.K.) of the SF apparatus, to the following equation for a single exponential process:

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

where $P_1 = |A_\infty - A_0|$, $P_2 = k_{\text{obs}}$ or k , and $P_3 = A_\infty$. Subsequent processing of kinetic results was carried out by using Sigmaplot 5.0 (Jandel Scientific, Erkrath, Germany).

Computer modelling of the intermediates formed by reaction of papain, caricain and actinidin with the thionoester substrate (4)

The starting coordinates for the three proteins with known structures were taken from the Protein Data Bank [21]: papain (9pap [22]), caricain (papaya proteinase Ω , 1ppo [23]) and actinidin (2act [24]). Buried water molecules (25 in papain, 24 in caricain and 20 in actinidin) were retained. All residues were assumed to be in their fully charged states: i.e. Arg, Lys and N-termini with charges of +1; Asp, Glu and C-termini with charges of –1; the catalytic site sidechains of Cys and His

Scheme 1 Synthesis of *N*-acetyl-Phe-Gly methyl thionoester (**4**)

with charges of -1 and $+1$ respectively, as required for the ion-pair state. The proteins were represented by the polar-hydrogen CHARMM force field [25,26], and the water molecules by the transferable intermolecular potential ('TIP3') [27]. The initial treatment of the structures was carried out as described for the modelling of two thiolsubtilisins [28]. The non-bonded interactions were truncated at $14 \text{ \AA}$, the electrostatic interactions at $12 \text{ \AA}$, and truncation of the van der Waals interactions was initiated at $8 \text{ \AA}$ and was complete by $12 \text{ \AA}$. The substrate coordinates 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. Electrostatic potentials mapped onto the molecular surfaces of the enzymes were displayed using GRASP [29].

The substrate was docked as the THI with the sulphur atom of Cys²⁵ bonded to the electrophilic carbon atom of the thionoester moiety. The geometry of the THI was optimized by using cycles of steepest-descent and adopted-basis-Newton-Raphson minimizations [25]. 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 three enzyme-substrate systems were each solvated with shells of water molecules $6 \text{ \AA}$ in thickness. The structures were again minimized to relieve clashes with the added water molecules, and the whole system was then subjected to a series of MD simulations at 300 K .

To investigate the release of methanol from the protein environment, the optimized THI structures were converted to the dithioacylenzyme intermediates and methanol. The geometries of these structures were optimized and subjected to MD simulations at 300 K , both in the absence and presence of bulk water, to investigate the diffusion of the methanol product.

RESULTS AND DISCUSSION

Synthesis of *N*-acetyl-Phe-Gly methyl thionoester (**4**)

The thionoester **4** was synthesized as indicated in Scheme 1. Aminoacetonitrile was allowed to react with Boc-Phe-*N*-hydroxysuccinimide ester to produce the P₁-P₂ amide bond required as a recognition feature for papain. After deprotection with formic acid and acetylation with acetic anhydride, the

resulting imidoester hydrochloride was produced from the nitrile by treatment with methanol and HCl gas. This was then dissolved in ice-cold methanolic pyridine and used to provide the methyl thionoester **4** by treatment with H₂S. The product **4** contains the major recognition features of a substrate for papain (a Phe residue at P₂ as an occupant for the S₂-subsite and the P₁-P₂ amide bond required to span the active centre cleft), and the thionoester group to permit spectroscopic detection of the dithioacylenzyme intermediate during catalysis.

Time courses of the acylation and deacylation phases of the catalysis of the hydrolysis of the thionoester substrate (**4**) by papain, caricain, actinidin and ficin

Examples of time courses for the hydrolysis of substrate **4** catalysed by the four cysteine proteinase variants with $[S]_0 \gg [E]_T$ are shown in Figure 2. As expected, in all cases, rapid formation of the dithioacylenzyme (ES'), detected by increase in A_{315} , is followed by slower loss of absorbance at this wavelength resulting from deacylation. These aspects of the results are in accord with the model of equation (1) when the formation of the first transient (the adsorptive complex, ES) is complete within the deadtime of the SF apparatus and the formation of the second transient (ES') is slow enough to be observed on the (shorter) SF-timescale. An exponential rise of [ES'] to its steady state value is followed by the rate-determining deacylation phase which occurs over a longer SF-timescale. The latter involves exponential decrease in [ES'], which is independent of $[S]_0$, and is characterized by a first-order rate constant k [k_{+3} of equation (1) and also equation (5), see below].

Characteristics of the deacylation phases

Analysis of the first-order decreases of A_{315} [such as those shown in Figure 3(b) for papain, and Figure 3(h) for actinidin] provided the following values of the rate constant, k (s⁻¹), for the reactions of the four dithioacylenzymes: papain, 0.78 ± 0.06 ; caricain, 0.11 ± 0.01 (results not shown); actinidin, 0.058 ± 0.002 ; ficin, 0.04 ± 0.002 (results not shown). In terms of the model of equation (1) values of k are those of k_{+3} , the rate constant for the hydrolysis of ES' (deacylation). The value of k for the papain

Figure 2 Formation [(a), (c), (e) and (g)] and subsequent deacylation [(b), (d), (f) and (h)] of the diithioacylzyme intermediates in the hydrolysis of *N*-acetyl-Phe-Gly methyl thionoester (4) catalysed by papain (4.65 μM) [(a) and (b)], caricain (1.90 μM) [(c) and (d)], actinidin (4.05 μM) [(e) and (f)] and ficin (1.45 μM) [(g) and (h)]

The SF-traces in ascending order in (a), (c), (e) and (g) and from left to right in (b), (d), (f) and (h) correspond to the following substrate concentrations (in μM): (a) and (b), 200, 300, 400; (c) and (d), 20, 50, 100; (e) and (f), 300, 400, 450; (g) and (h), 50, 100, 150, 200, 250. The experiments were carried out at 25 $^{\circ}\text{C}$, pH 5.22 and / 0.1, in 4.2% (v/v) aqueous dimethyl sulfoxide.

reaction is 20 times greater than that for the reaction of ficin, 13 times greater than that for the actinidin reaction and 7 times greater than that for the caricain reaction. Results presented below suggest that for the reactions of ficin and actinidin k may be characteristic, not of the hydrolysis of ES' , but rather, of a rate-determining conformational change in the $\text{ES}'\cdot\text{P}_1$ complex prior to deacylation. By contrast, no evidence was obtained to suggest that k might not characterize the hydrolysis of ES' [i.e. k_{+3} of equation (1)] for the reactions of papain and caricain. It is noteworthy that the substantially larger value of k for the reaction of the specific acylpapain intermediate generated in the present work by using *N*-acetylphenylalanylglycine methyl thionoester (4) relative to that for the reaction of the analogous actinidin intermediate was not found when using less specific thionoester substrates [5]. For example, loss of the dithioester

chromophore generated by using benzoylglycine methyl thionoester (1) as substrate was found to be characterized by the following values of k (s^{-1}) [5]: papain, 0.084 ± 0.002 ; actinidin, 0.104 ± 0.001 . The comparison of these sets of results for specific and less specific substrates suggests that binding interactions may play an important role in determining the nature of the rate-determining step in the cysteine proteinase catalytic mechanism.

Characteristics of the pre-steady state (acylation) phases of the hydrolysis of substrate (4) catalysed by the four enzymes

In terms of the model of equation (1), the observed first-order rate constant, k_{obs} , for the pre-steady state phase when the reaction is carried out with $[\text{S}]_0 \gg [\text{E}]_{\text{T}}$ [e.g. Figures 3(a) and 2(g)] is predicted to vary with $[\text{S}]_0$ according to equation (2) [16], where:

$$K_{\text{m(acyl)}} = (k_{-1} + k_{+2}) / k_{+1}$$

which approximates to $k_{-1}/k_{+1} = K_{\text{s}}$ when quasi-equilibrium around ES obtains ($k_{+2} \ll k_{-1}$, which is a necessary consequence of $k_{\text{cat}}/K_{\text{m}} \ll k_{+1}$ [30]).

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

Equation (2) predicts a hyperbolic dependence of $k_{\text{obs}} - k_{+3}$ on $[\text{S}]_0$. Figures 4(a) and 4(b) demonstrate such hyperbolic responses for the pre-steady state reactions of substrate 4 with papain and caricain respectively. The lack of a discernible intercept on the ordinate in both cases provides no evidence for a model more complex than equation (1), particularly the inclusion of significant reversibility of ES' formation.

Analysis of the hyperbolic curves in Figures 4(a) and 4(b) provides values of $K_{\text{m(acyl)}}$ ($183.5 \pm 52.9 \mu\text{M}$ for the papain reaction and $56.3 \pm 7.9 \mu\text{M}$ for the caricain reaction) and of k_{+2} ($116.2 \pm 13.5 \text{ s}^{-1}$ for the papain reaction and $18.4 \pm 0.6 \text{ s}^{-1}$ for the caricain reaction). The values of the individual rate constants (k_{+2} , k_{+3}) and of $K_{\text{m(acyl)}}$ permit the calculation of the values of the steady-state parameters defined by equations (3) and (4), and of their ratio, the specificity constant, $k_{\text{cat}}/K_{\text{m}} = k_{+2}/K_{\text{m(acyl)}}$ as follows: papain reaction, $k_{\text{cat}} = 0.77 \text{ s}^{-1}$, $K_{\text{m}} = 1.22 \mu\text{M}$, $k_{\text{cat}}/K_{\text{m}} = 6.3 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$; caricain reaction, $k_{\text{cat}} = 0.11 \text{ s}^{-1}$, $K_{\text{m}} = 0.34 \mu\text{M}$, $k_{\text{cat}}/K_{\text{m}} = 3.2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$.

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

$$K_{\text{m}} = K_{\text{m(acyl)}} \cdot \frac{k_{+3}}{k_{+2} + k_{+3}} \quad (4)$$

The pre-steady state parameters show that the 2-fold advantage of papain over caricain, in terms of the specificity constant, arises from a 6-fold advantage in the chemistry of the acylation step coupled with 3-fold weaker binding [assuming that $K_{\text{m(acyl)}} = K_{\text{s}}$].

In marked contrast to the hyperbolic saturation curves for papain and caricain (Figures 4a and 4b), the plots of k_{obs} versus $[\text{S}]_0$ for actinidin (Figure 4c) and for ficin (Figure 4d) are linear. Linear plots are predicted from equation (2) $[\text{S}]_0 \ll K_{\text{m(acyl)}}$, and such plots were also found for papain and caricain when reactions of thionoester substrates that lack formal recognition features were studied (e.g. [5,31,32]). The particularly noteworthy features of Figures 4(c) and 4(d) are the values of the ordinate intercept (Figure 4c, 2.3 s^{-1} for actinidin; Figure 4d, 0.77 s^{-1} for ficin), which are much greater than the values assumed to be

Figure 3 Demonstration of the adherence to first-order kinetics of the acylation [(a), (c), (e), (g), (i) and (k)] and deacylation [(b), (d), (f), (h), (j) and (l)] phases of the hydrolysis of *N*-acetyl-Phe-Gly methyl thionoester (4) catalysed by papain (4.65 μ M) (a)–(f) and actinidin (4.05 μ M) (g)–(l)

For the papain reaction $[S]_0 = 50 \mu$ M, for the actinidin reaction $[S]_0 = 300 \mu$ M and other reaction conditions were as described in Figure 2. (a), (b), (g) and (h) show the fits of the SF kinetic data (noisy signals) to single exponentials (smooth curves), and (c), (d), (i) and (j) are the associated residuals. (e), (f), (k) and (l) demonstrate the linearity of the corresponding first-order logarithmic plots to 93%, 85%, 91% and 77% reaction respectively. The lines are linear regression lines.

those of k_{+3} in terms of the model of equation (1) determined independently (0.058 s^{-1} for actinidin and 0.04 s^{-1} for ficin). The discrepancies between the intercept values and the values assumed to be those of k_{+3} may be accounted for by acknowledging the reversibility of ES' formation such that the model of equation (1) becomes that of equation (5).

For this model k_{obs} is given by equation (6) [33], which becomes equation (7) when $[S]_0 \ll K_{\text{m(acyl)}}$.

$$k_{\text{obs}} = \frac{k_{+2}[S]_0}{K_{\text{m(acyl)}} + [S]_0} + k_{-2} + k_{+3} \quad (6)$$

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

Figures 4(c) and 4(d) could be accounted for by equation (7) in which the intercept values are those of $k_{-2} + k_{+3}$ (i.e. greater than those of k_{+3} alone), and the values of the slopes are those of $k_{+2}/K_{\text{m(acyl)}}$ ($1.27 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the actinidin reaction, and $2.92 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the ficin reaction). The intercept values of Figures 4(c) and 4(d) and the values of k_{+3} from Figure 3 provide the values of k_{-2} as 2.24 s^{-1} for the actinidin reaction and 0.73 s^{-1} for the ficin reaction.

The problem with the model of equation (5), however, 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 $ES' \cdot P$ complex has a life-time long enough to permit intracomplex reaction of ES' with P_1 to reform ES . There is no reason to suppose that the chromophore with λ_{max} about 315 nm, characteristic of the dithioester moiety of ES' , might be significantly perturbed by the presence of methanol (P_1) in the complex. A necessary aspect of this hypothesis is that release of P_1 into bulk solvent is a slow process. Slow diffusion of methanol out of the protein seems unlikely, however, and the suggestion by Hollaway and Hardman [16] of a slow conformational change is an attractive alternative. This (probably subtle) change in conformation is envisaged to permit rapid desorption of methanol and its replacement by the water molecule required to hydrogen bond to (His¹⁶¹)-imidazole (actinidin numbering) as part of the deacylation process. The simplest model incorporating this slow conformational change is equation (8) in which $(ES' \cdot P_1)^*$ represents the conformation of the acylenzyme-methanol complex from which methanol diffuses rapidly to leave $(ES')^*$, which binds water and undergoes deacylation.

Figure 4 Dependence on substrate concentration of the observed first-order rate constant (k_{obs}) for the pre-steady state phase of the hydrolysis of *N*-acetyl-Phe-Gly methyl thionoester (4) catalysed by papain (a), caricain (b), actinidin (c) and ficin (d)

The enzyme concentrations and other reaction conditions are as described in Figure 2. The experimental points are mean values for three determinations. The S.D. values were at most $\pm 10\%$ of the means. The results are plotted as $k_{\text{obs}} - k_{+3}$ versus $[S]_0$ in (a) and (b), and as k_{obs} versus $[S]_0$ in (c) and (d). In (a) and (b) the lines are theoretical for the appropriate transform of equation (2) and the following parameter values: (a) $K_{\text{m(acyl)}} = 183.5 \pm 52.9 \mu\text{M}$ and $k_{\text{obs}}^{\text{lim}} = 116.2 \pm 13.5 \text{ s}^{-1}$; (b) $K_{\text{m(acyl)}} = 56.3 \pm 7.9 \mu\text{M}$ and $k_{\text{obs}}^{\text{lim}} = 18.4 \pm 0.6 \text{ s}^{-1}$. In (c) and (d) the lines are theoretical for equation (7) and the following parameter values: (c) $k_{+2}/K_{\text{m(acyl)}} = (1.27 \times 10^4) \pm (0.1 \times 10^4) \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{-2} + k_{+3} = 2.3 \pm 0.2 \text{ s}^{-1}$, from which $k_{-2} = 2.2 \text{ s}^{-1}$; (d) $k_{+2}/K_{\text{m(acyl)}} = (2.92 \times 10^4) \pm (0.1 \times 10^4) \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{-2} + k_{+3} = 0.77 \pm 0.45 \text{ s}^{-1}$, from which $k_{-2} = 0.73 \text{ s}^{-1}$.

In terms of equation (8) 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 $(\text{ES}')^*$. If the conformational change in $\text{ES}' \cdot \text{P}_1$ (k_{+3}) is rate-determining, this would account for the simultaneous release of P_1 and P_2 in the catalysis by ficin deduced by Hollaway and Hardman [16]. It may be that following the release of P_1 from $(\text{ES}')^*$, the conformation of the acylenzyme returns to that of ES' prior to hydrolysis.

Investigation of the interactions of papain, caricain and actinidin by computer modelling

The simplest interpretation of the kinetic results discussed above is that, in the reactions catalysed by ficin and actinidin, the rate-determining step is the change in conformation of $\text{ES}' \cdot \text{P}_1$ [k_{+3} of equation (8)], whereas in the reactions catalysed by papain and caricain, the rate-determining step is the hydrolysis of ES' [k_{+3} of equation (1), or k_{+5} of equation (8)]. In an attempt to account for the differences in the kinetics of the two pairs of enzyme variants, the structures of papain, caricain and actinidin were examined, and models of complexes of these enzymes with the thionoester substrate 4 at the THI and dithioacylzyme stages of the acylation process were constructed. The crystal structure of ficin has not been reported, and the lack of a sequence precluded construction of a structure by homology modelling.

Figure 5 Illustration of the movement of Met²¹¹ in actinidin consequent on docking of the substrate 4 at the THI stage of catalysis

The broken line ($\text{M}^{211\text{s}}$) shows the orientation of Met²¹¹ at the start of the MD simulation with the thioether sidechain pointing into the S_2 -subsite towards the $\text{Cys}^{25}\text{-His}^{159}$ ion pair. During the simulation, as docking occurs, this sidechain moves out of the binding pocket to the position labelled $\text{M}^{211\text{f}}$ where it points towards Asn¹¹⁵ (see text for details).

Two aspects of the reactions were addressed by modelling (i) interactions in the S_2 -subsites, the major recognition site, and (ii) the fates of the first product (methanol) following its release from the THIs consequent on dithioacylzyme formation.

Binding in the S_2 -subsites

This was investigated because one possible reason for the differential behaviour of the leaving group might relate to differences in the binding modes of the substrate in actinidin and ficin, as opposed to those in papain and caricain. The initial minimized states of all three THIs are similar in the catalytic-site regions. Thus the S atom of the substrate moiety accepts hydrogen bonds from the backbone N-H of Cys²⁵ and the sidechain N-H of Gln¹⁹, as might be expected from the crystal structures of papain inhibited by substrate analogues [3]. In addition, a sidechain N-H of His¹⁵⁹ donates a hydrogen bond to the oxygen atom of the methoxy-leaving group, and Sy of Cys²⁵ accepts a hydrogen bond from the backbone N-H of Ala¹⁶⁰. Away from the catalytic site, in the intermediates formed by papain and caricain, the backbone C=O of Asp¹⁵⁸ and the backbone N-H and C=O of Gly⁶⁶ make hydrogen bonds with the $\text{P}_1\text{-P}_2$ amide bond of the substrate moiety, and the sidechain of the Phe residue of the substrate occupies the hydrophobic pocket of the S_2 -subsite. These interactions also are in agreement with those shown crystallographically by Drenth et al. [3]. It is

Figure 6 Electrostatic and binding site characteristics of papain, caricain and actinidin

Top panels show 'snapshots' from MD simulations carried out on the solvated THI intermediates formed from substrate **4** and papain (**a**), caricain (**c**) and actinidin (**e**). Colour codes: magenta (substrate); for the protein, C (green), H (white), S (yellow), N (blue) and O (red). The broken lines represent the hydrogen bonds formed between the THI and the protein. In the minimized state the Phe ring of the substrate is contained in the S_2 -subsites of papain (**a**) and caricain (**c**) between the backbone atoms of Val¹⁵⁷ and Asp¹⁵⁸ and the sidechain atoms of Pro⁶⁸, Tyr⁶⁹, Val¹³³ and Val¹⁵⁷. In the case of actinidin, the thioether sidechain of Met²¹¹ points into the active centre (Figure 5, M^{211s}) and prevents the Phe sidechain from entering by 0.7 Å less than in papain and caricain. During the MD simulation, the movement of Met²¹¹ shown in Figure 5 (from M^{211s} to M^{211t}) allows the Phe sidechain to fit into the S_2 -subsite of actinidin as shown in (**e**). Bottom panels show the electrostatic potentials ranging from -10 kT (red) to +10 kT (blue) mapped onto the molecular surfaces of papain (**b**), caricain (**d**) and actinidin (**f**). The uncharged regions are shown in grey.

well known that actinidin binds hydrophobic substrates with less affinity than papain (for example [34]). This is usually attributed to the fact the residue in position 211 at the end of the S_2 -subsite is methionine in actinidin, as opposed to serine in papain (residue 205). In caricain also, the analogous residue is serine (residue 209). In the crystal structure of actinidin [34] the thioether sidechain of Met²¹¹ stretches across the end of the binding pocket, making it noticeably shorter. It was not surprising, therefore, that in the initial stage of the modelling, uniquely, substrate **4** did not fit well into the S_2 -subsite of actinidin. An unexpected finding was that the situation changed markedly when the MD simulations were carried out. Whereas in the papain and caricain systems all interactions of enzyme and substrate were maintained, in the actinidin system a large movement of the sidechain of Met²¹¹ was observed. This is shown in Figure 5. In the starting position (labelled M^{211s}) the sidechain points towards the Cys²⁵-His¹⁵⁹ ion pair. During the simulation, however, as part of the binding process it moves out of the binding pocket and points towards Asn¹¹⁵. This movement allows the Phe sidechain of the substrate to fit well into the S_2 -subsite of actinidin, as in the case for papain and caricain (Figures 6a, 6c and 6e). The sidechain of Met²¹¹ remains pointing out of the binding pocket during the rest of the simulation. The inference that binding of the substrate can induce this change in the conformation of Met²¹¹ suggests that the presence of this residue might not, after all, be the main reason for the weaker binding of hydrophobic substrates to actinidin.

Another major difference between papain and the other two enzymes that were modelled is the much greater electrostatic potential on the surfaces of actinidin (negative) and caricain (positive) than is the case for papain (Figures 6b, 6d and 6f). We have shown previously [35] that it is the large negative potential on actinidin that prevents its isolation by covalent chromatography when using a negatively-charged spacer derived from glutathione, a procedure that was originally successfully developed for papain [36]. Our preliminary steady state kinetic data for the hydrolysis of *N*-benzoyl-Phe-Val-Arg-4-nitroanilide and *N*-acetyl-Phe-Leu-4-nitroanilide catalysed by papain and actinidin (T. S. F. Bailey, E. W. Thomas and K. Brocklehurst, unpublished work) suggest that binding of these substrates containing extensive hydrophobic areas is substantially weaker for actinidin than for papain, irrespective of whether there is a cationic sidechain at the P₁ position. This finding, together with the modelling described above, suggest that the reason for the weak binding of hydrophobic substrates to actinidin might have an electrostatic component, rather than a purely steric cause due to the presence of Met²¹¹ in the S_2 -binding site. The reason for the lack of analogous repulsion by caricain which has substantial surface potential (albeit positive) requires more detailed study, particularly to investigate specific microsite effects.

Fates of the methanol product following its release from the THIs

This was studied by MD simulation which provided a possible explanation for the accumulation of methanol specifically in the

Figure 7 MD simulations demonstrating the difference in the fates of the methanol (P_1) product from reaction of substrate (**4**) with papain (**a**) and actinidin (**b**)

(**a**) The papain C^α -backbone is represented by the thin line, Cys²⁵ acylated by substrate **4** and His¹⁵⁹ by the thick lines labelled **C** and **H**, respectively, and methanol molecules by unlabelled thick lines. The papain backbone and the acyl group of the substrate are shown only once, but the positions of the diffusing methanol molecule are taken from successive frames of an MD simulation each separated by 2 ps. (**b**) The analogous actinidin system, except that the two 'snapshots' of the 140–160-residue region demonstrate the highly correlated movement of the region associated with the Asp¹⁴²–Lys¹⁴⁵ ion pair over ca. 4.8 Å from the positions labelled **D** and **K** (bounded by a thick continuous line) to those labelled **D₁** and **K₁** (bounded by a thick broken line). Several positions of the methanol molecule, which appears to be entrapped within this region, are shown as unlabelled short thick lines.

actinidin reaction and, thus, for the observed reversibility of dithioacylenzyme formation. General acid catalysis by the catalytic site imidazolium cation is expected to result in the formation of methanol initially hydrogen-bonded to the neutral imidazole group. After minimization, the methanol is found, in 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 diffuses rapidly (over < 8 ps) out of papain and caricain by various pathways (e.g. Figure 7a). In marked contrast, however, the methanol molecule 'lingers' around the active centre of actinidin (Figures 7b and 8), particularly in the

region encompassing, Lys¹⁴⁵–His¹⁶² and Phe¹⁴⁴, Trp¹⁸⁴ and Trp¹⁸⁸. Uniquely in actinidin, Lys¹⁴⁵ makes a salt bridge with Asp¹⁴² (and an additional hydrogen bond to the backbone carbonyl oxygen atom of Ala¹⁴⁰), the analogous interactions in papain (Lys¹³⁹ and Gln¹⁴²) and in caricain (Arg¹³⁹ and Gln¹⁴²) being much weaker. In actinidin the region between residues 140–146, 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) in a highly correlated manner which appear to control the motions of the methanol molecule. It seems possible that the changes in the cavity leading to rapid release of the methanol into bulk solvent might be those suggested to be rate-determining in the model of equation (8) $[ES' \cdot P_1 \rightarrow (ES' \cdot P_1)^*]$.

CONCLUSION

The marked differences in behaviour between papain and actinidin in the acylation and post-acylation processes of the catalytic act, demonstrated here kinetically, augment the growing awareness of differences in active-centre chemistry revealed by thiol-targeted reactivity probes, enantiomeric substrates and the pH-dependence of k_{cat}/K_m . Indeed of the members of the papain family investigated by such methods, papain and actinidin display the most disparate reactivity characteristics, with caricain and ficin occupying intermediate positions in the series. Examples include the responses to anionic reactivity probes [37], binding site–catalytic site signalling in reactions of substrate-derived 2-pyridyl disulphide reactivity probes [11,38,39] and its interplay with electrostatic fields [13,14], and P_2 – S_2 stereochemical selectivity [15,40]. The MD simulations suggest a structural interpretation of the difference in the catalytic characteristics of papain and actinidin demonstrated in the present work. The fact that the behaviour of ficin resembles that of actinidin, rather than that of papain, predicts the possible existence of a motif in ficin analogous to the cavity that appears to rely on the Asp¹⁴²–Lys¹⁴⁵ interaction in actinidin.

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Figure 8 MD simulation of the acylactinidin intermediate–methanol complex illustrating the diffusion of the methanol molecule (**M**) from the vicinity of His¹⁶² (**a**) to the cavity formed by Phe¹⁴⁴, Lys¹⁴⁵, Trp¹⁸⁴ and Trp¹⁸⁸ (**b**)

The two MD 'snapshots', (**a**) and (**b**), represent the two extremes of the progress of the methanol molecule from the region of its formation during acylation of Cys²⁵ by substrate **4** to the cavity in which it is retained prior to diffusion to bulk solvent.

REFERENCES

- Brocklehurst, K., Watts, A. B., Patel, M., Verma, C. and Thomas, E. W. (1998) Cysteine proteinases. In *Comprehensive Biological Catalysis*. (Sinnott, M. L., ed.), vol. 1, pp. 381–423. Academic Press, London.
- Henes, J. B., Mattis, J. A. and Fruton, J. S. (1979) Interaction of papain with derivatives of phenylalanyl glycine: fluorescence studies. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1131–1134.
- Drenth, J., Kalk, K. H. and Swen, H. M. (1976) Binding of chloromethyl ketone substrate analogues to crystalline papain. *Biochemistry* **15**, 3731–3738.
- Lowe, G. and Williams, A. (1965) Direct evidence for an acylated thiol as an intermediate in papain- and ficin-catalysed hydrolysis. *Biochem. J.* **96**, 189–193.
- Brocklehurst, K., Carey, P. R., Lee, H., Salih, E. and Storer, A. C. (1984) Comparative resonance Raman spectroscopic and kinetic studies of acylenzymes involving papain, actinidin and papaya peptidase II. *Biochem. J.* **223**, 649–657.
- Tonge, P. J., Menard, R., Storer, A. C., Ruzsicska, B. P. and Carey, P. R. (1991) Markedly different acyl papain structures deacylate at similar rates: Resonance Raman spectroscopic and kinetic evidence. *J. Am. Chem. Soc.* **113**, 4297–4303.
- Pinitglang, S., Watts, A. B., Patel, M., Reid, J. D., Noble, M. A., Gul, S., Bokth, A., Naeem, A., Patel, H., Thomas, E. W. et al. (1997) A classical enzyme active centre motif lacks catalytic competence until modulated electrostatically. *Biochemistry* **36**, 9968–9982.
- Noble, M. A., Gul, S., Verma, C. S. and Brocklehurst, K. (2000) Ionization characteristics and chemical influences of aspartic acid residue 158 of papain and caricain determined by structure-related kinetic and computational techniques: multiple electrostatic modulators of active-centre chemistry. *Biochem. J.* **351**, 723–733.
- Szawelski, R. J. and Wharton, C. W. (1981) Kinetic solvent isotope effects on the deacylation of specific acyl-papains. *Biochem. J.* **199**, 681–692.
- Welsh, W. J. and Lin, Y. (1997) Discussion of the catalytic pathway of cysteine proteases based on AM1 calculations. *J. Mol. Struct. (THEOCHEM)* **401**, 315–326.
- Brocklehurst, K., Brocklehurst, S. M., Kowlessur, D., O'Driscoll, M., Patel, G., Salih, E., Templeton, W., Thomas, E., Topham, C. M. and Willenbrock, F. (1988) Supercrystallographic resolution of interactions contributing to enzyme catalysis by use of natural structural variants and reactivity probe kinetics. *Biochem. J.* **256**, 543–555.
- Kowlessur, D., Topham, C. M., Thomas, E. W., O'Driscoll, M., Templeton, W. and Brocklehurst, K. (1989) Identification of signalling and non-signalling binding contributions to enzyme reactivity. Alternative combinations of binding interactions provide for change in transition state geometry in reactions of papain. *Biochem. J.* **258**, 755–764.
- Kowlessur, D., O'Driscoll, M., Topham, C. M., Templeton, W., Thomas, E. W. and Brocklehurst, K. (1989) 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. *Biochem. J.* **259**, 443–452.
- Topham, C. M., Salih, E., Frazao, C., Kowlessur, D., Overington, J. P., Thomas, M., Brocklehurst, S. M., Patel, M., Thomas, E. W. and Brocklehurst, K. (1991) Structure–function relationships in the cysteine proteinases actinidin, papain, and papaya proteinase Ω deduced by knowledge-based modelling and active centre characteristics determined by two-hydronic-state reactivity probe kinetics of catalysis. *Biochem. J.* **280**, 79–92.
- Patel, M., Kayani, S., Templeton, W., Mellor, G. W., Thomas, E. W. and Brocklehurst, K. (1992) Evaluation of hydrogen bonding and enantiomeric P_2-S_2 hydrophobic contacts in dynamic aspects of molecular recognition by papain. *Biochem. J.* **287**, 881–889.
- Hollaway, M. R. and Hardman, M. J. (1973) Evidence for a rate-limiting conformation change in the catalytic steps of the ficin and papain-catalysed hydrolysis of benzoyloxycarbonyl-L-lysine *p*-nitrophenyl ester. *Eur. J. Biochem.* **32**, 537–546.
- Brocklehurst, K., Baines, B. S. and Malthouse, J. P. G. (1981) Differences in the interactions of the catalytic groups of the active centres of actinidin and papain. Rapid purification of fully active actinidin by covalent chromatography and characterization of its active centre by use of two-protonic-state reactivity probes. *Biochem. J.* **197**, 739–746.
- Malthouse, J. P. G. and Brocklehurst, K. (1976) Preparation of fully active ficin by covalent chromatography and characterization of its active centre using 2,2'-dipyridyl disulphide as a reactivity probe. *Biochem. J.* **159**, 221–234.
- Baines, B. S. and Brocklehurst, K. (1979) A necessary modification to the preparation of papain from any high quality latex of *Carica papaya* and evidence for the structural integrity of the enzyme produced by traditional methods. *Biochem. J.* **177**, 541–548.
- Baines, B. S. and Brocklehurst, K. (1978) A spectrophotometric method for the detection of contaminant chymopapains in preparation of papain. Selective modification of one type of thiol group in the chymopapains by a two-protonic-state reagent. *Biochem. J.* **178**, 345–347.
- Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N. and Bourne, P. E. (2000) The Protein Data Bank. *Nucleic Acids Res.* **28**, 235–242.
- Kamphuis, I. G., Kalk, K. H., Swarte, M. B. A. and Drenth, J. (1984) Structure of papain refined at 1.65 Å resolution. *J. Mol. Biol.* **179**, 233–256.
- Pickersgill, R. W., Rizkallah, P., Harris, G. W. and Goodenough, P. W. (1991) Determination of the structure of papaya protease Ω . *Acta Crystallogr.* **B47**, 766–771.
- Baker, E. N. and Dodson, E. J. (1980) Crystallographic refinement of the structure of actinidin at 1.7 Å resolution by fast fourier least-squares methods. *Acta Crystallogr.* **A36**, 559–572.
- Brooks, B. R., Brucoleri, R. E., Olafson, B. D., States, D. J., Swaminathan, S. and Karplus, M. (1983) CHARMM – a program for macromolecular energy, minimization and dynamics calculations. *J. Comput. Chem.* **4**, 165–175.
- Neria, E., Fischer, S. and Karplus, M. (1996) Simulation of activation free energies in molecular systems. *J. Chem. Phys.* **105**, 1902–1921.
- Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W. and Klein, M. L. (1983) Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* **79**, 926–935.
- Plou, F. J., Kowlessur, D., Malthouse, J. P. G., Mellor, G. W., Hartshorn, M. J., Pinitglang, S., Patel, H., Topham, C. M., Thomas, E. W., Verma, C. and Brocklehurst, K. (1996) Characterization of the electrostatic perturbation of a catalytic site (Cys)–S[−]/(His)–Im⁺H ion-pair in one type of serine proteinase architecture by kinetic and computational studies on chemically-mutated subtilisin variants. *J. Mol. Biol.* **257**, 1088–1111.
- Nicholls, A., Sharp, K. A. and Honig, B. (1991) Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins* **11**, 281–296.
- Brocklehurst, K. (1979) The equilibrium assumption is valid for the kinetic treatment of most time-dependent protein modification reactions. *Biochem. J.* **181**, 775–778.
- Storer, A. C. and Carey, P. R. (1985) Comparison of the kinetics and mechanism of the papain catalyzed hydrolysis of esters and thionoesters. *Biochemistry* **24**, 6808–6818.
- Storer, A. C., Angus, R. H. and Carey, P. R. (1988) Comparison of the kinetics of the papain-catalyzed hydrolysis of glycine- and alanine-based esters and thiono esters. *Biochemistry* **27**, 264–268.
- Johnson, K. A. (1986) Rapid kinetic analysis of mechanochemical adenosine triphosphatases. *Methods Enzymol.* **134**, 677–705.
- Baker, E. N., Boland, M. J., Calder, P. C. and Hardman, M. J. (1980) The specificity of actinidin and its relationship to the structure of the enzyme. *Biochim. Biophys. Acta* **616**, 30–34.
- Thomas, M. P., Verma, C., Boyd, S. M. and Brocklehurst, K. (1995) The structural origins of the unusual specificities observed in the isolation of chymopapain M and actinidin by covalent chromatography and the lack of inhibition of chymopapain M by cystatin. *Biochem. J.* **306**, 39–46.
- Brocklehurst, K., Carlsson, J., Kierstan, M. P. J. and Crook, E. M. (1973) Covalent chromatography: preparation of fully active papain from dried papaya latex. *Biochem. J.* **133**, 573–584.
- Brocklehurst, K., Mushiri, S. M., Patel, G. and Willenbrock, F. (1983) A marked gradation in active-centre properties in the cysteine proteinases revealed by neutral and anionic reactivity probes. Reactivity characteristics of the thiol groups of actinidin, ficin, papain and papaya peptidase A towards 4,4'-dipyridyl disulphide and 5,5'-dithiobis-(2-nitrobenzoate)dianion. *Biochem. J.* **209**, 873–879.
- Salih, E., Malthouse, J. P. G., Kowlessur, D., Jarvis, M., O'Driscoll, M. and Brocklehurst, K. (1987) Differences in the chemical and catalytic characteristics of two crystallographically 'identical' enzyme catalytic sites. Characterisation of actinidin and papain by a combination of pH-dependent substrate catalysis kinetics and reactivity probe studies targeted on the catalytic-site thiol group and its immediate microenvironment. *Biochem. J.* **247**, 181–193.
- Patel, M., Thomas, M. P., Kayani, I. S., Mellor, G. W., Thomas, E. W. and Brocklehurst, K. (1993) Ficin: a cysteine proteinase with binding site–catalytic site signalling characteristics intermediate between those of papain and actinidin. *Biochem. Soc. Trans.* **21**, 216S.
- Patel, M., Saleem, I., Mellor, G. W., Sreedharan, S., Templeton, W., Thomas, E. W., Thomas, M. and Brocklehurst, K. (1992) Variation in the P_2-S_2 stereochemical selectivity towards the enantiomeric *N*-acetylphenylalanylglycine 4-nitroanilides among the cysteine proteinases papain, ficin and actinidin. *Biochem. J.* **281**, 553–559.