Temperature-dependences of the kinetics of reactions of papain and actinidin with a series of reactivity probes differing in key molecular recognition features

Sheraz GUL^{*1}, Geoffrey W. MELLOR^{*2}, Emrys W. THOMAS[†] and Keith BROCKLEHURST^{*3}

^{*}Laboratory of Structural and Mechanistic Enzymology, School of Biological and Chemical Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, U.K., and [†]Department of Biological Sciences, University of Salford, The Crescent, Salford M5 4JW, U.K.

The temperature-dependences of the second-order rate constants (k) of the reactions of the catalytic site thiol groups of two cysteine peptidases papain (EC 3.4.22.2) and actinidin (EC 3.4.22.14) with a series of seven 2-pyridyl disulphide reactivity probes (R-S-S-2-Py, in which R provides variation in recognition features) were determined at pH 6.7 at temperatures in the range 4–30 °C by stopped-flow methodology and were used to calculate values of $\Delta S^\ddagger$, $\Delta H^\ddagger$ and $\Delta G^\ddagger$. The marked changes in $\Delta S^\ddagger$ from negative to positive in the papain reactions consequent on provision of increase in the opportunities for key non-covalent recognition

interactions may implicate microsite desolvation in binding site–catalytic site signalling to provide a catalytically relevant transition state. The substantially different behaviour of actinidin including apparent masking of changes in $\Delta H^\ddagger$ by an endothermic conformational change suggests a difference in mechanism involving kinetically significant conformational change.

Key words: actinidin, activation parameter, conformational change, microsite desolvation, papain, temperature-dependence.

INTRODUCTION

The least well understood aspects of enzyme catalysis and active-centre chemistry include dynamic aspects of non-covalent interactions that are involved in substrate and inhibitor recognition and their effects on catalytic site reactivity characteristics. Members of the papain family of cysteine peptidases have proved to be useful in contributing to the detection and understanding of these phenomena (see below).

Cysteine peptidases are endopeptidases whose catalytic activity relies on the thiol group of a cysteine residue (for a comprehensive review, see [1], and for more recent reviews on their catalytic mechanism and active-centre chemistry, see [2,3]). These enzymes are widely distributed in animals, plants, bacteria and viruses, and constitute a superfamily composed of six enzyme families [4]. Most of the well-characterized and intensively studied cysteine peptidases are members of the papain family. Those that have been subjected to detailed structural and mechanistic study contain a Cys-S[−]/His-Im⁺H (imidazolium) ion pair as the major feature of the catalytic site. The thiolate anion component of this interactive system becomes transiently acylated during catalysis, assisted by general acid-catalysed expulsion of the leaving group from a tetrahedral species, the acid catalysis being provided by the Im⁺H component.

Papain (EC 3.4.22.2) was long considered to be the cysteine peptidase archetype, and mechanistic studies were concentrated largely on this member of the family for many years. Studies that included the use of natural variants of papain, however, led to the discovery of considerable variation in catalytic site reactivity characteristics within the family. These differences demonstrate that the traditional view of the archetypal nature of papain is correct only for low-resolution aspects of structure–function relation-

ships such as the central mechanistic roles of the common Cys²⁵-S[−]/His¹⁵⁹-Im⁺H ion pair (papain numbering) and that much is to be gained by study of natural variants. Particularly noteworthy variation exists in (i) the discrepancy between the pK_a values of -S[−]/Im⁺H ion-pair formation and the essentially common pK_a value for the development of catalytic activity ($k_{\text{cat}}/K_{\text{m}}$) leading to the important concept of an essential electrostatic modulator, (ii) the coupling of key non-covalent molecular recognition interactions with catalytic site chemistry and their interplay with electrostatic effects, and (iii) both postacylation- and free enzyme–protein dynamics. As a result of these differences, gradations in functional characteristics and mechanistic phenomena that are more easily revealed in some members of the family than in others were identified. Papain exhibits characteristics at one end of a spectrum of chemical behaviour with actinidin (EC 3.4.22.14) at the other. Caricain [papaya (*Carica papaya*) proteinase Ω , EC 3.4.22.30] and ficin (EC 3.4.22.3) each exhibit behaviour intermediate between these extremes.

Many of the advances indicated above were obtained by using substrate-derived 2-pyridyl disulphide time-dependent inhibitors as two-protonic state reactivity probes [5] and pH-dependent kinetics [6]. In the present study, the temperature-dependences of the kinetics of selected cysteine peptidase–probe reactions were determined to ascertain whether changes in transition geometry occasioned by some of the specific non-covalent binding interactions and identified by the use of our probes [2,3] might be accompanied by selective active-centre desolvation. The crystal structures of papain [7] and actinidin [8] each reveal an ordered network of water molecules in their active centres and some binding interactions might be predicted to interrupt these networks. The present paper reports the activation parameters ($\Delta S^\ddagger$ and $\Delta H^\ddagger$) for the reactions of papain and actinidin with a series of

Abbreviations used: Im⁺H, imidazolium; 2-Py, 2-pyridyl; SF, stopped-flow.

¹ Present address: Assay Development, GlaxoSmithKline Research & Development Ltd, New Frontiers Science Park (North), Third Avenue, Harlow, Essex CM19 5AW, U.K.

² Present address: Screening and Compound Profiling, Glaxo Medicines R&D Centre, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, U.K.

³ To whom correspondence should be addressed (email kb1@qmul.ac.uk).

Figure 1 Structures of the reactivity probes used in the temperature-dependent kinetic studies

2-pyridyl disulphide reactivity probes in which individual specific non-covalent binding sites are introduced in various combinations into the probe molecule. The striking changes in $\Delta S^\ddagger$ from large and negative to large and positive in the reactions of papain, which are mirrored only partially in the reactions of actinidin, and the enthalpy–entropy relationships for the reactions of both enzymes are discussed and interpreted in terms of selective active-centre desolvation and, in the case of actinidin, masking of decrease in $\Delta H^\ddagger$ by an endothermic conformational change, possibly related to those deduced by using a specific thionoester substrate.

MATERIALS AND METHODS

Enzymes and reactivity probes

Methods of purification of actinidin [9] and papain [10], which include the production of fully active enzymes by covalent chromatography (reviewed in [11]), have been described previously, as have their active-centre content and evaluation by spectroscopic titration at 343 nm ($\epsilon_{343} = 8080 \text{ M}^{-1} \cdot \text{cm}^{-1}$) using 2,2'-dipyridyl disulphide as titrant [12].

The syntheses and characterization of the seven reactivity probes used in the present work (shown in Figure 1) have been described previously in connection with pH-dependence studies: ethyl 2-pyridyl disulphide (1) [13]; 2-(acetoxymethyl)ethyl 2'-pyridyl disulphide (2) [14]; 2-(acetamidomethyl)ethyl 2'-pyridyl disulphide (3) [15]; 2-(*N'*-acetyl-L-phenylalanyl)hydroxyethyl 2'-pyridyl disulphide (4) [10] and its D-enantiomer (5) [16]; 2-(*N'*-acetyl-L-phenylalanyl)aminoethyl 2'-pyridyl disulphide (6) [15] and its D-enantiomer (7) [17].

SF (stopped-flow) kinetics

Kinetic studies on the reactions of the catalytic site thiol groups of papain and actinidin with the reactivity probes were performed by using a Hi-Tech Scientific SF spectrophotometer, kinetics workstation, and data acquisition and analysis software. Monochromator entrance and exit slit widths were set at 0.5 mm. The sample handling unit was fitted with a UG5 bandpass filter to eliminate stray light and was configured for thermostatically con-

trolled temperature cycling. Temperature control was achieved using a Grant LTD6 water bath.

Reactions were carried out in the low-temperature coefficient ($\text{dp}K_a/\text{dT} = 0.0028$ [18]) $\text{KH}_2\text{PO}_4/\text{NaOH}$ buffer, pH 6.7, I 0.1 M, at ten different temperatures in the range 4–30 °C under (pseudo) first-order conditions with $[\text{probe}] \geq 20$ times $[\text{enzyme thiol}]$. The stock solution of the phosphate buffer was prepared at I 0.3 M and was diluted to provide a solution containing the reactivity probe at I 0.1 M in one of the syringes of the SF spectrophotometer. The other syringe contained the enzyme in 0.1 M KCl. Mixing of equal volumes of these two solutions maintained I 0.1 M in the reaction chamber. The pH was checked to be 6.7 in the effluent from the reaction chamber and in dummy reaction mixtures. The release of pyridine-2-thione was monitored at 343 nm. Values of the observed first-order rate constants (k_{obs}) were obtained by fitting the equation for a single exponential process,

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

where $P_1 = A_\infty - A_0$, $P_2 = k_{\text{obs}}$ and $P_3 = A_\infty$, to the absorbance (A) – time (t) data collected as multiple superimposable traces by the SF system.

Determination of activation parameters

Values of entropy of activation ($\Delta S^\ddagger$) and enthalpy of activation ($\Delta H^\ddagger$) were determined by linear regression of the left-hand side of eqn (1) on $1000/T$, where T is the absolute temperature and 1000 is a convenient scaling factor:

$$R [\ln(k/T) - \ln(k_B/h)] = \Delta S^\ddagger - \Delta H^\ddagger/T \quad (1)$$

Eqn (1) is a convenient transform of the Eyring equation (see the Results and Discussion section) in which R is the gas constant ($8.13145 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$), k_B is Boltzmann's constant, h is Planck's constant and the value of $\ln(k_B/h)$ is 23.76. The regression provides $-\Delta H^\ddagger$ as the slope of the linear plot and $\Delta S^\ddagger$ as the ordinate intercept.

RESULTS AND DISCUSSION

Temperature-dependent kinetics: aspects of analysis

The well-known Arrhenius and Eyring equations used in chemical kinetics can be properly applied to reactions of enzymes and sensibly interpreted providing that: (i) a restricted temperature range that avoids denaturation (often taken to be 0–50 °C) is used, (ii) account is taken of the temperature-dependence of acid dissociation constants, and (iii) the mechanistic significance of the rate parameter is known and lacking in complexity. In the present study, it was possible to: (i) restrict the temperature range to 4–30 °C, where the enzymes are conformationally stable without detriment to the analysis, (ii) study the reactions at pH 6.7, a value around which the rate constants change only slightly with change in pH in the range ~6–8, and (iii) determine the second-order rate constant (k) for the enzyme–probe reactions whose mechanistic significance (reaction of the catalytic site cysteinyl thiol group with the neutral probe molecule) is well defined and is the same for each probe.

For many reactions, the increase in k with increase in temperature is described by the Arrhenius equation (eqn 2) where E_a , the activation energy, in terms of transition state theory, is equal to $\Delta H^\ddagger + RT$.

$$k = A e^{-E_a/RT} \quad (2)$$

Table 1 The activation parameters ($\Delta H^\ddagger$ and $\Delta S^\ddagger$) for the reactions of papain and actinidin with 2-pyridyl disulphide reactivity probes

The values $\Delta G^\ddagger$ were calculated at 298 K from $\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$; the standard errors relate to the values of the ordinate intercept ($\Delta S^\ddagger$) and the slope ($-\Delta H^\ddagger$) of the linear plots typified by that in Figure 2 analysed by using eqn (1).

Probe	Papain			Actinidin		
	$\Delta H^\ddagger$ (kJ · mol ⁻¹)	$\Delta S^\ddagger$ (J · mol ⁻¹ · K ⁻¹)	$\Delta G^\ddagger$ (kJ · mol ⁻¹)	$\Delta H^\ddagger$ (kJ · mol ⁻¹)	$\Delta S^\ddagger$ (J · mol ⁻¹ · K ⁻¹)	$\Delta G^\ddagger$ (kJ · mol ⁻¹)
CH ₃ -CH ₂ -S-S-2-Py (1)	28 ± 1.2	-79.9 ± 4.0	51.8	25.1 ± 2.1	-112.1 ± 6.9	58.5
CH ₃ CO-O-[CH ₂] ₂ -S-S-2-Py (2)	32 ± 1.2	-60.5 ± 4.0	50.0	46.5 ± 1.2	-42.4 ± 3.8	59.1
CH ₃ CO-NH-[CH ₂] ₂ -S-S-2-Py (3)	37.6 ± 0.6	-37.1 ± 2.0	48.7	47.6 ± 1.9	-34.5 ± 6.3	57.9
CH ₃ CO-[L-Phe]-O-[CH ₂] ₂ -S-S-2-Py (4)	43.1 ± 1.2	18.7 ± 4.1	37.5	41.6 ± 2.4	-21.4 ± 8.0	48.0
CH ₃ CO-[D-Phe]-O-[CH ₂] ₂ -S-S-2-Py (5)	38.6 ± 1.3	-19.0 ± 4.4	44.3	47.3 ± 1.3	-21.0 ± 4.5	53.6
CH ₃ CO-[L-Phe]-NH-[CH ₂] ₂ -S-S-2-Py (6)	52.4 ± 2.0	57.7 ± 7.0	35.2	49.7 ± 3.7	12.1 ± 1.2	46.1
CH ₃ CO-[D-Phe]-NH-[CH ₂] ₂ -S-S-2-Py (7)	45.8 ± 1.6	11.0 ± 5.5	42.5	47.1 ± 1.0	-18.2 ± 3.3	52.5

Transition state theory leads to the Eyring equation (eqn 3, in which $\Delta G^\ddagger$ is the free energy of activation) or eqn (4) and division throughout of eqn (4) by T leads to the logarithmic form, eqn (5).

$$k = (\mathbf{k}_B/h) T e^{-\Delta G^\ddagger/RT} \quad (3)$$

$$k = (\mathbf{k}_B/h) T e^{-\Delta H^\ddagger/RT} \cdot e^{\Delta S^\ddagger/R} \quad (4)$$

$$\ln(k/T) = \ln(\mathbf{k}_B/h) - \Delta H^\ddagger/RT + \Delta S^\ddagger/R \quad (5)$$

The form of the Eyring equation (eqn 1, see the Materials and methods section) convenient for analysis is obtained from eqn (5) by multiplying through by and collecting terms in R . Regression analysis provides values of $\Delta S^\ddagger$ and $\Delta H^\ddagger$ directly.

Despite the well-known long extrapolation to 0 K required to obtain the value of $\Delta S^\ddagger$ and the relatively large standard errors on this parameter, conclusions drawn from temperature-dependence studies that, as in the present study, rely on the sign of $\Delta S^\ddagger$ (positive or negative) and not only on its magnitude are usually not affected by the error inherent in the extrapolation process (see, for example, [19]).

Dependence on non-covalent interactions of $\Delta S^\ddagger$ and $\Delta H^\ddagger$ for the reactions of papain and actinidin with the 2-pyridyl disulphide reactivity probes 1–7 at pH 6.7

The values of $\Delta S^\ddagger$ and $\Delta H^\ddagger$ for the two sets of enzyme–probe reactions, together with those of $\Delta G^\ddagger$ at 298 K calculated as $\Delta H^\ddagger - T\Delta S^\ddagger$, are collected in Table 1, and a typical linear plot derived from eqn (1) (for the reaction of papain with the substrate-analogue probe **6**) is shown in Figure 2. The variations, particularly in $\Delta S^\ddagger$, for the reactions of papain are considered first and then compared with those for the reactions of actinidin.

Reactions of papain

For the reactions of papain with the ‘featureless’ probe, CH₃-CH₂-S-S-2-Py (where 2-Py is 2-pyridyl) (**1**), $\Delta S^\ddagger$ has a large negative value (-79.9 ± 4.0 J · mol⁻¹ · K⁻¹), as expected, because of the restriction of the motion of the probe in the transition state, and the probable minimal disruption of the water network in the active centre of the enzyme owing to the lack of recognition features in the small alkyl group of **1**. When provision is made for the possibility first of one hydrogen bond [between the backbone N-H of Gly⁶⁶ and the carbonyl O of CH₃CO-O-[CH₂]₂-S-S-2-Py (**2**)] and then also of a second hydrogen bond [between the backbone carbonyl O of Asp¹⁵⁸ and the N-H of the amide group of CH₃CO-NH-[CH₂]₂-S-S-2-Py (**3**)], the values of $\Delta S^\ddagger$ become progressively less negative (from $(-79.9 \pm 4.0$ to -60.5 ± 4.0 to -37.1 ± 2.0 J · mol⁻¹ · K⁻¹). In these three cases, the negative values of $\Delta S^\ddagger$ are characteristic of transition states that are more

Figure 2 Determination of the activation parameters ($\Delta H^\ddagger$ and $\Delta S^\ddagger$) for the reaction of papain with 2-(*N*-acetyl-L-phenylalanyl-amino)ethylene 2'-pyridyl disulphide (**6**)

The points are experimental and the continuous line was obtained by linear regression. Values of rate constants (k) obtained from the analysis of multiple (≥ 3) superimposable SF kinetic runs were used to calculate the left-hand side of eqn (1) using values of the parameters R , $\mathbf{k}_B$ and h given in the Materials and methods section. Extrapolation of the line to zero on the abscissa provides $\Delta S^\ddagger$ as the ordinate intercept; the slope provides $-\Delta H^\ddagger$.

ordered than the corresponding ground states. The progressive decrease in their negativity could be accounted for by the increased probability of an appropriate transition-state geometry. The changes in transition-state geometry consequent on provision of various recognition interactions in these reactions may be inferred from marked changes in shapes of pH- k profiles [16,17].

The most striking changes in $\Delta S^\ddagger$ arise when the probe is equipped with phenylalanine at P₂ as a potential occupant for the major recognition site for papain, the hydrophobic S₂ subsite. For the reaction of CH₃CO-[L-Phe]-NH-[CH₂]₂-S-S-2-Py (**6**), the probe containing both a P₁-P₂ amide bond and L-phenylalanine at P₂, which has a pH- k profile with a shape strikingly similar to that of a pH- k_{cat}/K_m profile, i.e. maximal activity around pH 6, $\Delta S^\ddagger$ has become large and positive ($+57.7 \pm 7.0$ J · mol⁻¹ · K⁻¹). The effectiveness of this reaction at pH values around 6 suggest a high probability of a well-organized transition state in which the thiolate component of the catalytic site ion pair and the Im⁺H component are correctly positioned to act as nucleophile and hydrogen bond donor respectively. The fact that this reaction is characterized also by a large positive entropy of activation,

however, suggests a third component which provides for a less-ordered structure in the transition state than in the ground state. The most obvious candidate for this additional component is the water network of the active centre. This leads to the view that desolvation resulting from binding of the substrate-like probe **6** in the transition state conformation with consequent release of bound water molecules allows both components of the ion pair to be involved synchronously in the reaction, and is most likely to be the cause of the large positive value of $\Delta S^\ddagger$.

The effect on $\Delta S^\ddagger$ of removing the possibility of hydrogen bonding of the probe to the backbone carbonyl O of Asp¹⁵⁸ while retaining L-phenylalanine at P₂ was investigated by comparison of the value for the reaction of CH₃CO-[L-Phe]-O-[CH₂]₂-S-S-2-Py (**4**) with that for the reaction of probe **6**. Removal of the possibility of the (P₁)-NH $\cdots$ O=C < (Asp¹⁵⁸) hydrogen bond results in a substantial decrease in the value of $\Delta S^\ddagger$ from $+57.7 \pm 7.0$ to $+18.7 \pm 4.1$ J $\cdot$ mol⁻¹ $\cdot$ K⁻¹. The fact that $\Delta S^\ddagger$ for the reaction of probe **5**, the D-enantiomer of **4** (-19.0 ± 4.4 J $\cdot$ mol⁻¹ $\cdot$ K⁻¹) is substantially less negative than that for the reaction of probe **2** which lacks the D-phenylalanine moiety (-60.5 ± 4.0 J $\cdot$ mol⁻¹ $\cdot$ K⁻¹) provides evidence for the binding of (P₂)-D-phenylalanine to papain (see [17] for a discussion of this phenomenon deduced by model building). It is noteworthy that $\Delta S^\ddagger$ for the reaction of probe **5** is still negative and becomes positive for the D-enantiomer only when the possibility of hydrogen bonding to Asp¹⁵⁸ is restored in probe **7**. This supports the conclusion suggested by the relative values of difference kinetic specificity energies [16], that transition state geometry in reactions of papain is determined by interdependent binding interactions in the S₂-subsite, S₁-S₂ intersubsite region and catalytic site. The present study also suggests that selective desolvation of the regions of the active centre participates in the binding site-catalytic site coupling process.

Reactions of actinidin

$\Delta S^\ddagger$ for the reactions of the featureless probe (**1**) is even more negative (-112.1 ± 6.9 J $\cdot$ mol⁻¹ $\cdot$ K⁻¹) than for the analogous reaction of papain (-79.9 ± 4.0 J $\cdot$ mol⁻¹ $\cdot$ K⁻¹), possibly indicating even more restriction of the motion of the probe in the transition state. As in the case of the papain reactions, incorporation of the P₁-P₂ hydrogen bonding systems in the P₁-P₂ ester probe (**2**) and the P₁-P₂ amide probe (**3**) results in the progressive decrease in the negativity of $\Delta S^\ddagger$ from -112.1 ± 6.9 to -42.2 ± 3.8 to -34.5 ± 6.3 J $\cdot$ mol⁻¹ $\cdot$ K⁻¹, presumably reflecting the increased probability of binding with appropriate orientation in the transition state. Although the probability of an appropriate transition-state geometry is increased by the opportunity to form the additional trans-cleft hydrogen bond, the mechanism of the reaction of actinidin is different from that of papain. Thus, whereas for papain there is substantial reaction involving activation of the leaving group by hydrogen bond donation by the Im⁺H component of the catalytic site ion pair, the reaction of actinidin is mainly with the formally protonated probe ($pK_a \sim 2.5$). This conclusion arises from the observation of maximal activity at pH 6 for the papain reaction, but at pH 3–4 for the actinidin reaction (see [16,17]).

A particularly marked difference between the reactions of actinidin and papain occurs when the hydrogen bond acceptor (the ester carbonyl O in probe **2**) is augmented by (P₂)-L-phenylalanine in probe **4**. In the papain reaction, this addition results in $\Delta S^\ddagger$ changing from markedly negative (-60.5 ± 4.0 J $\cdot$ mol⁻¹ $\cdot$ K⁻¹) to substantially positive ($+18.7 \pm 4.1$ J $\cdot$ mol⁻¹ $\cdot$ K⁻¹). In the actinidin reaction, however, it remains substantially negative (-21.4 ± 8.0 J $\cdot$ mol⁻¹ $\cdot$ K⁻¹), although less negative than the value for the amide probe **3** devoid of the L-phenylalanine moiety. This suggests that, although binding of the L-phenylalanine moiety contributes

to a correct transition-state geometry for nucleophilic attack of the thiolate anion of actinidin, desolvation is not as effective as in the papain reaction. Another striking difference between the reactions of the two enzymes is that changing the chirality of the phenylalanine moiety from L-phenylalanine in **4** to D-phenylalanine in **5** results in a marked change in $\Delta S^\ddagger$ from $+18.7 \pm 4.1$ J $\cdot$ mol⁻¹ $\cdot$ K⁻¹ to -19.0 ± 4.4 J $\cdot$ mol⁻¹ $\cdot$ K⁻¹ for the papain reaction, but essentially no change for the actinidin reaction (both approx. -21 J $\cdot$ mol⁻¹ $\cdot$ K⁻¹). This suggests that binding either of the enantiomeric phenylalanine moieties to actinidin does not achieve a good fit of probes **4** and **5** in the hydrophobic pocket of the S₂ subsite. This is in accord with the prediction from the crystal structure of actinidin [8] which reveals a shorter pocket than that in papain [7]. A significantly better fit is achieved for the L-phenylalanine enantiomer; however, when provision is made also for the (P₁) > N-H $\cdots$ O=C < (Asp¹⁶¹) hydrogen bond by changing the P₁-P₂ ester bond in **4** to the P₁-P₂ amide bond in **6**. Reaction of actinidin with **6** is characterized by a positive $\Delta S^\ddagger$ ($+12.1 \pm 1.2$ J $\cdot$ mol⁻¹ $\cdot$ K⁻¹), indicating more effective desolvation, although less effective than in the papain reaction ($\Delta S^\ddagger = +57.7 \pm 7.0$ J $\cdot$ mol⁻¹ $\cdot$ K⁻¹). The change in structure from the L-phenylalanine enantiomer **6** to the D-enantiomer **7** which results in the decrease in $\Delta S^\ddagger$ from $+57.7 \pm 7.0$ to $+11.0 \pm 5.5$ J $\cdot$ mol⁻¹ $\cdot$ K⁻¹ for the papain reaction, results in a similar change for the actinidin reaction, in this case from $+12.1 \pm 1.2$ to -18.2 ± 3.3 J $\cdot$ mol⁻¹ $\cdot$ K⁻¹. In both cases, therefore, the binding mode of the D-phenylalanine moiety differs from that of the L-phenylalanine moiety with respect to the (P₂)-binding pocket. This contrasts with the lack of dependence on P₂ chirality for actinidin when the possibility of the (P₁) > N-H $\cdots$ O=C < (Asp¹⁵⁸) hydrogen bond is missing (in probes **4** and **5**). This emphasizes the interdependence of P₂/S₂ subsite and P₁-P₂/S₁-S₂ intersubsite binding.

Differences in the relative changes in $\Delta H^\ddagger$ and $\Delta S^\ddagger$ that characterize reactions of papain and actinidin with the substrate-derived reactivity probes 2–7

Linear relationships between enthalpies and entropies occur widely in molecular associations particularly those involving hydrogen bonding (see, for example, [20–22]). Whether such relationships arise at least in part from chemical causation (extrathermodynamic relationships, isokinetic effect, enthalpy–entropy compensation effect) as against a dominant statistical compensation pattern has been the subject of much debate (see, for example, [22] and references therein). The analysis of enthalpy–entropy compensation reported by Cornish-Bowden [23] necessitates extreme caution in discussion of this phenomenon. In the present paper, we report the differences observed in the temperature-dependent kinetic characteristics of some analogous reactions of papain and actinidin that are influenced by non-covalent interactions and point out possible correlations with our results from other types of kinetic study reported previously.

Linear dependence of $\Delta H^\ddagger$ on $\Delta S^\ddagger$ was observed for the reactions of papain with the substrate-derived probes 2–7 (plot not shown; data in Table 1). Evidence for chemical causation suggested by Krug et al. [24,25] includes a substantial difference between the compensation temperature, T_c (the approximate value of which is provided by the slope of the $\Delta H^\ddagger$ against $\Delta S^\ddagger$ plot) and the average experimental temperature, and a linear relationship between $\Delta H^\ddagger$ and $\Delta G^\ddagger$. These two criteria appear to be met in the present work ($T_c = 200$ K, average temperature 295 K; linear plot not shown, results in Tables 1 and 2).

Such chemical causation as may exist in enthalpy–entropy compensation is generally considered to be at least partly due to

Table 2 Illustration of the different dependences of $\Delta G^\ddagger$ on $\Delta H^\ddagger$ for the reactions of papain and actinidin with substrate-derived reactivity probes 2–7

The formulae of the probes are given in Table 1 and their structures in Figure 1.

Probe	$\Delta G^\ddagger/\Delta H^\ddagger$	
	Papain	Actinidin
2	1.56	1.27
3	1.30	1.22
5	1.15	1.13
7	0.93	1.11
4	0.87	1.15
6	0.67	0.93

the perturbation, release or change in the state of water consequent upon ligand binding by the protein. This may reflect the responsiveness of proteins to ligand binding [26], and the compensation effect for the reactions of papain correlates well with the responsiveness of papain to changes in the structure of the substrate-derived probes 2–6 detected by the changes in the pH–*k* profiles [16,17]. The much lower sensitivity of the compensation effect for the corresponding reactions of actinidin (Table 1) and the clustering of the $\Delta G^\ddagger/\Delta H^\ddagger$ data (Table 2) contrast markedly with the results for papain and correlate with the relative insensitivity of the pH–*k* profiles for the actinidin reactions to respond to structural change in the probes. The absence of substantial differences in $\Delta H^\ddagger$ for the reactions of the substrate-derived probes with actinidin could result from the masking of an enzyme-mediated decrease in enthalpy of activation by an endothermic conformational change as suggested by Jencks [27] for the deacylation of a series of acyl α -chymotrypsins. Such an interpretation of the temperature-dependent kinetic data for the reactions of actinidin might be related to the kinetically significant change(s) in conformation detected uniquely for actinidin by using a specific thionoester substrate [28,29].

We thank the Engineering and Physical Sciences Research Council and the Biotechnology and Biological Sciences Research Council for support of this work, including postdoctoral research assistantships for S. G. and G. W. M. respectively.

REFERENCES

- 1 Brocklehurst, K., Willenbrock, F. and Salih, E. (1987) Cysteine proteinases. In *Hydrolytic Enzymes* (Neuberger, A. and Brocklehurst, K., eds.), pp. 39–158, Elsevier, Amsterdam
- 2 Brocklehurst, K., Watts, A. B., Patel, M., Verma, C. S. and Thomas, E. W. (1998) Cysteine proteinases. In *Comprehensive Biological Catalysis* (Sinnott, M. L., ed.), vol. 1, pp. 381–423, Academic Press, London
- 3 Hussain, S., Khan, A. and Brocklehurst, K. (2002) A review of developments in the study of cysteine proteinase mechanism: opportunities for the investigation of electrostatic effects and dynamic aspects of molecular recognition in enzyme chemistry. *Recent Res. Dev. Biochem.* **3**, 653–677
- 4 Barrett, A. J., Rawlings, N. D. and Woessner, Jr, J. F. (eds.) (2004) *Handbook of proteolytic enzymes*, Elsevier, London
- 5 Brocklehurst, K. (1982) Two-protonic-state electrophiles as probes of enzyme mechanisms. *Methods Enzymol.* **87C**, 427–469
- 6 Brocklehurst, K. (1994) A sound basis for pH-dependent kinetic studies on enzymes. *Protein Eng.* **7**, 291–299
- 7 Kamphius, I. G., Kalk, K. H., Swarte, M. B. A. and Drenth, J. (1984) Structure of papain refined at 1.6 Å resolution. *J. Mol. Biol.* **179**, 233–256
- 8 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. Sect. A Found. Crystallogr.* **36**, 559–572
- 9 Brocklehurst, K., Baines, B. S. and Malthouse, J. P. (1981) Differences in the interaction 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
- 10 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
- 11 Brocklehurst, K., Courey, A. J., Gul, S., Lin, S. and Moritz, R. L. (2004) Affinity and immunoaffinity chromatography. In *Purifying Proteins for Proteomics: A Laboratory Manual* (Simpson, R. J., ed.), pp. 221–273, Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- 12 Baines, B. S. and Brocklehurst, K. (1978) A spectrophotometric method for the detection of contaminant chymopapains in preparations of papain: selective modification of one type of thiol group in the chymopapains by a two-protonic-state reagent. *Biochem. J.* **173**, 345–347
- 13 Brocklehurst, K., Kowlessur, D., Patel, G., Templeton, W., Quigley, K., Thomas, E. W., Wharton, C. W., Willenbrock, F. and Szawelski, R. J. (1988) Consequences of molecular recognition in the S₁–S₂ intersubsite region of papain for catalytic-site chemistry: change in pH-dependence characteristics and generation of an inverse solvent kinetic isotope effect by introduction of a P₁–P₂ amide bond into a two-protonic-state reactivity probe. *Biochem. J.* **250**, 761–772
- 14 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) Supracrystallographic resolution of interactions contributing to enzyme catalysis by use of natural structural variants and reactivity-probe kinetics. *Biochem. J.* **256**, 543–555
- 15 Brocklehurst, K., Kowlessur, D., O'Driscoll, M., Patel, G., Quenby, S., Salih, E., Templeton, W., Thomas, E. W. and Willenbrock, F. (1987) Substrate-derived two-protonic-state electrophiles as sensitive kinetic specificity probes for cysteine proteinases: activation of 2-pyridyl disulphides by hydrogen-bonding. *Biochem. J.* **244**, 173–181
- 16 Patel, M., Kayani, I. S., Templeton, W., Mellor, G. W., Thomas, E. W. and Brocklehurst, K. (1992) Evaluation of hydrogen-bonding and enantiomeric P₂–S₂ hydrophobic contacts in dynamic aspects of molecular recognition by papain. *Biochem. J.* **287**, 881–889
- 17 Templeton, W., Kowlessur, D., Thomas, E. W., Topham, C. M. and Brocklehurst, K. (1990) A re-appraisal of the structural basis of stereochemical recognition in papain. Insensitivity of binding-site–catalytic-site signalling to P₂-chirality in a time-dependent inhibition. *Biochem. J.* **266**, 645–651
- 18 Stevens, L. (1996) Solutions used in enzymology. In *Enzymology Labfax* (Engel, P. C., ed.), pp. 269–274, Academic Press, London
- 19 Narlikar, G. J. and Herschlag, D. (1996) Isolation of a local tertiary folding transition in the context of a globally folded RNA. *Nat. Struct. Biol.* **3**, 701–710
- 20 Searle, M. S. and Williams, D. H. (1993) On the stability of nucleic acid structures in solution: enthalpy–entropy compensations, internal rotations and reversibility. *Nucleic Acids Res.* **21**, 2051–2056
- 21 Gilli, P., Ferretti, V., Gilli, G. and Borea, P. A. (1994) Enthalpy–entropy compensation in drug–receptor binding. *J. Phys. Chem.* **98**, 1515–1518
- 22 Dunitz, J. D. (1995) Win some, lose some: enthalpy–entropy compensation in weak intermolecular interactions. *Chem. Biol.* **2**, 709–712
- 23 Cornish-Bowden, A. (2002) Enthalpy–entropy compensation: a phantom phenomenon. *J. Biosci.* **27**, 121–126
- 24 Krug, R. R., Hunter, W. G. and Greiger, R. A. (1976) Enthalpy–entropy compensation. 1. Some fundamental statistical problems associated with the analysis of van't Hoff and Arrhenius data. *J. Phys. Chem.* **80**, 2335–2340
- 25 Krug, R. R., Hunter, W. G. and Greiger, R. A. (1976) Enthalpy–entropy compensation. 2. Separation of the chemical from the statistical effect. *J. Phys. Chem.* **80**, 2341–2351
- 26 Eftink, M. R., Anusiem, A. C. and Biltonen, R. L. (1983) Enthalpy–entropy compensation and heat capacity changes for protein–ligand interactions: general thermodynamic models and data for the binding of nucleotides to ribonuclease A. *Biochemistry* **22**, 3884–3896
- 27 Jencks, W. P. (1969) *Catalysis*. In *Chemistry and Enzymology*, pp. 282–320, McGraw-Hill Book Company, New York
- 28 Reid, J. D., Hussain, S., Sreedharan, S. K., Bailey, T. S., Pinitglang, S., Thomas, E. W., Verma, C. S. and Brocklehurst, K. (2001) Variation in aspects cysteine proteinase mechanism deduced by spectroscopic observation of dithioester intermediates, kinetic analysis and molecular dynamics simulations. *Biochem. J.* **357**, 343–352
- 29 Reid, J. D., Hussain, S., Bailey, T. S., Sonkaria, S., Sreedharan, S. K., Thomas, E. W., Resmini, M. and Brocklehurst, K. (2004) Isomerization of the uncomplexed actinidin molecule: kinetic accessibility of additional steps in enzyme catalysis provided by solvent perturbation. *Biochem. J.* **378**, 699–703