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The interplay of electrostatic and binding interactions determining active centre chemistry and catalytic activity in actinidin and papain

The structural variation within the cysteine proteinase family provides possibilities for investigation of the consequences of different electrostatic effects and of different binding opportunities for active centre chemistry and catalytic mechanism (Brocklehurst *et al.*, 1987*b*). Papain (EC 3.4.22.2) and actinidin (EC 3.4.22.14) are of particular interest because, although their crystal structures are closely similar (Kamphuis *et al.*, 1985), evidence for substantial differences in the characteristics and behaviour of their catalytic sites continues to accumulate (Salih *et al.*, 1987; Brocklehurst *et al.*, 1988*b*, and references therein).

Pickersgill *et al.* (1988) calculated the electrostatic fields of actinidin and papain by the finite-difference procedure of Warwicker & Watson (1982) and suggest that the differences in electrostatic potential may explain the different reactivity characteristics without the need to invoke conformational or mechanistic differences. These calculations further define the electrostatic differences deduced from earlier studies (see below) but do not reveal the variety of factors that contribute to the reactivity characteristics of these enzymes. The principal purpose of the present Letter is to point out evidence in the literature, apparently not considered by Pickersgill *et al.* (1988), and from unpublished work, which suggests that catalytic site reactivity in actinidin and papain is determined by a complex interplay of electrostatic effects and binding interactions. In addition, it seems helpful to point out that the sites in papain identified by Pickersgill *et al.* (1988) as targets for change by site-directed mutagenesis may not be appropriate.

The catalytic activity of these (and analogous) enzymes does not depend in a simple way merely on the production of the $-S^-/-ImH^+$ catalytic site ion pair as Pickersgill *et al.* (1988) appear to have assumed. Thus the pH–activity profiles are not bell-shaped with each characterized by only two pK_a values (see Salih *et al.*, 1987, and references therein). Rather, for papain, the increase in $k_{cat.}/K_m$ with increase in pH occurs across two positively co-operative hydronic dissociations each of pK_a approx. 4, and for actinidin the increase in $k_{cat.}/K_m$ occurs in two distinct stages, one across $pK_a < 4$ (probably 3.1, the pK_a of $-SH/-ImH^+ \rightleftharpoons -S^-/ImH^+ + H^+$) and the other across pK_a 5.5. In actinidin at least, therefore, the existence of $-S^-/-ImH^+$ is not a sufficient condition for the full expression of activity. This dependence on an additional hydronic dissociation of pK_a 5–5.5 occurs also with cathepsin H and particularly with cathepsin B where it is both an absolute requirement for catalytic activity towards Z-Arg-Arg-NNap, despite the formation of the $-S^-/-ImH^+$ state across pK_a approx. 3, and a factor affecting thiol reactivity (see Willenbrock & Brocklehurst, 1986, and references therein), a phenomenon confirmed by Pohl *et al.* (1987) and by Polgar & Csoma (1987) who discuss possible electrostatic mechanisms.

Evidence that geometries of transition states in reactions involving the catalytic site ion pair of papain are markedly affected by binding interactions both in the hydrophobic pocket of the S_2 subsite and in the S_1–S_2 intersubsite region has come from kinetic studies using substrate-derived 2-pyridyl disulphides (e.g. Quigley *et al.*, 1986; Brocklehurst *et al.*, 1987*a*, 1988*a*,*b*). It is a particularly striking observation that binding of a P_1–P_2 amide bond across the interdomain cleft results in a substantial contribution from an $-ImH^+$-assisted transition state for the papain reactions (with k optimal at pH 6–7) but not for those of actinidin (with k minimal at pH 6–7), despite the virtually superimposable crystal structures in regions in and close to the catalytic sites of these enzymes. Another striking observation is that for the reactions of papain with the probes that do not possess a P_1–P_2 amide bond [compounds (I)–(III)], k increases with pH in alkaline media across pK_a 9.5, whereas for those that do [compounds (IV) and (V)] the corresponding decrease in k occurs mainly across pK_a 8.2: CH_3-CH_2-CH_2-S-S-2-Py (I), CH_3-CO-O-CH_2-CH_2-S-S-Py (II), CH_3-NH-CO-CH_2-CH_2-S-S-2-Py (III), CH_3-CO-NH-CH_2CH_2-S-S-2-Py (IV), CH_3-CO-NH-CH-(CH_2Ph)-CO-NH-CH_2-CH_2-S-S-2-Py (V). This change in pK_a from 9.5 to 8.2 with change in probe structure does not occur for the corresponding reactions of actinidin.

The simplest interpretation of these findings is that the pK_a manifested in reactions of papain that lack specific binding interactions (9.5) characterizes $-S^-/-ImH^+ \rightleftharpoons -S^-/-Im + H^+$ while the pK_a manifested when binding interactions permit operation of the $-ImH^+$-assisted transition state (8.2) is that of some other group controlling the binding or its signalling to the catalytic site, although a reversal of these assignments must remain a possibility. In reactions with other thiol-specific reagents (see e.g. Brocklehurst *et al.*, 1987*b*) observations of apparently single pK_a values between 8.2 and 9.5 might be due to influence of both ionizations with particular ratios of rate constants for the different reactive states (see Brocklehurst *et al.*, 1983, for an analysis of apparent variability in free enzyme pK_a values).

The pK_a value of 8.2 suggests a histidine imidazolium ion in a perturbing environment, and it may be relevant that papain contains two histidine residues, the catalytic site His-159 and His-81, whereas the only histidine residue in actinidin (His-162) is in the catalytic site (the sequence-aligned residue analogous to His-81 being Asn-84). His-81 appears to form an ion pair with a buried carboxylate anion (Glu-52).

The kinetic studies show that the reactivity charac-

teristics of the -S⁻/-ImH⁺ ion pair of papain are determined by a more complex array of factors than the immediate electrostatic field to which the catalytic site is exposed and that the situation in actinidin is quite different (see Brocklehurst *et al.*, 1988*b*, for detailed analysis). Observation of the kinetically influential pK_a of 8.2 in reactions of papain with compounds (IV) and (V) but not with compounds (I)–(III) led us to examine the pH-dependence of $k_{cat.}/K_m$ for papain-catalysed hydrolysis in alkaline media. Existing data (e.g. Lowe & Yuthavong, 1971; Mole & Horton, 1973) show that $k_{cat.}/K_m$ falls across pK_a 8.2 and suggest the possible existence of another pK_a approx. 9.5. More extensive kinetic data (C. M. Topham & K. Brocklehurst, unpublished work) demonstrate convincingly the existence of two kinetically influential ionizations with pK_a values of approx. 8 and 9.5 in the pH–$k_{cat.}/K_m$ profile for the papain-catalysed hydrolysis of L-Bz-Arg-NH-Np, a situation that applies also to the pH–k profile for reaction with compound (V) (Brocklehurst *et al.*, 1988*b*).

In the case of actinidin, the group associated with pK_a 5.0–5.5 whose state of ionization modulates both catalytic activity and reactivity of the -S⁻/ImH⁺ ion pair remains to be identified, as do two other groups, of pK_a values approx. 6.3 and 7 respectively, that affect ion pair reactivity when opportunities for certain of the specific binding interactions exist. The importance of the differences in the electrostatic fields to which the active centre regions of actinidin and papain are exposed in determining their characteristics has been examined previously both by experiment (see Brocklehurst *et al.*, 1987*b*) and by calculation (e.g. Lavery *et al.*, 1983) and has been discussed by a number of authors (see Baker & Drenth, 1987; Brocklehurst *et al.*, 1987*b*, for reviews). In our view the unique pH-dependent kinetic behaviour of actinidin might involve juxtaposed carboxy groups, with at least one unique to actinidin, possibly in a hydrophobic environment. Potential candidates that fulfil these criteria are to be found in Baker's (1980) description of the actinidin structure including a network predicted to have one pK_a of approx. 6 as was found for one of the paired carboxy groups (Glu-35) in lysozyme (Parsons & Raftery, 1970), and these would be rational choices for change by site-directed mutagenesis. It would be valuable also to effect changes in papain to provide negative charges that might influence the active centre region, analogous to those that exist already in actinidin, as well as changes involving His-81 (e.g. to Asn) to test the double ion-pair hypothesis (Brocklehurst *et al.*, 1988*b*). Pickersgill *et al.* (1988) suggest changing residues 118 and 135 in papain from glutamine to glutamic acid to test their electrostatic model. This suggestion does not seem appropriate, however, in view of the nucleotide sequence reported by Cohen *et al.* (1986) in which the codon for glutamic acid was found at amino acid positions 118 and 135 (and 47) rather than that for glutamine.

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Keith BROCKLEHURST,* Mairead O'DRISCOLL,*
Devanand KOWLESSUR,* Ian R. PHILLIPS,*
William TEMPLETON,* Emrys W. THOMAS,†
Christopher M. TOPHAM* and
Christopher W. WHARTON‡

*Department of Biochemistry, St. Bartholomew's Hospital Medical College, University of London, Charterhouse Square, London EC1M 6BQ, U.K., †Department of Biological Sciences, University of Salford, Salford M5 4WT, U.K., and ‡Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

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Structural and electrostatic differences between actinidin and papain account for differences in activity

Brocklehurst *et al.* (1989) discuss the evidence for substantial differences in the characteristics of the active sites of actinidin and papain and describe the interplay of electrostatic and binding interactions. In addition, these authors suggest that residues identified by Pickersgill *et al.* (1988) may not be appropriate targets for modification by site-directed mutagenesis. The purpose of this Letter is to present the electrostatic differences, which we suggest are primarily responsible for the different characteristics