Differences in the chemical and catalytic characteristics of two crystallographically 'identical' enzyme catalytic sites

Characterization 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

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The characteristics of actinidin (EC 3.4.22.14) and papain (EC 3.4.22.2), two cysteine proteinases whose catalytic-site regions appear to superimpose to a degree that approaches atomic co-ordinate accuracy of both crystal structures, were evaluated by determining (a) the pH-dependence in acid media of the acylation process of the catalytic act (k_{+2}/K_s) using *N*^α-benzoyl-L-arginine *p*-nitroanilide (L-Bz-Arg-Nan) as substrate and (b) the sensitivity of the reactivity of the catalytic-site thiol group and its pH-dependence to structural change in small, thiol-specific, two-protonic-state reactivity probes (2,2'-dipyridyl disulphide and methyl 2-pyridyl disulphide) where enzyme-probe contacts should be restricted to areas close to the catalytic site. Distortion of the catalytic sites of the two enzymes at pH < 4 was evaluated over time-scales appropriate for both stopped-flow reactivity probe kinetics (≤ 1–2 s) and steady-state substrate catalysis kinetics (3–5 min) by using the 2,2'-dipyridyl disulphide monocation as a titrant for non-distorted catalytic sites. This permitted a lower pH limit to be defined for valid kinetic analysis of both types. The behaviour of the enzymes at pH < 4 requires a kinetic model in which the apparently bimolecular reaction of enzyme with probe reagent is separated from the process leading to loss of conformational integrity by a potentially reversible step. The acylation of actinidin with L-Bz-Arg-Nan in acidic media occurs in two protonic states, one produced by raising the pH across $pK_a < 4$ which probably characterizes the formation of $-S^-/-ImH^+$ ion pair (pK_a approx. 3) and the other, of higher reactivity, produced by raising the pH across pK_a 5.5, which may characterize rearrangement of catalytic-site geometry. The pH-dependence of the acylation of papain by L-Bz-Arg-Nan is quite different and is not influenced by protonic dissociation with pK_a values in the range 5–6. The earlier conclusion that the acylation of papain depends on two protonic dissociations each with pK_a approx. 4 was confirmed. This argument is now more firmly based because titration with 2,2'-dipyridyl disulphide permits the loss of conformational integrity to be taken into account in the analysis of the kinetic data at very low pH. Methyl 2-pyridyl disulphide was synthesized by reaction of pyridine-2-thione with methyl methanethiolsulphonate and its pK_a at $I = 0.1$ was determined by spectral analysis at 307 nm to be 2.8. The pH-dependences of the apparent second-order rate constants (k) for the reactions of actinidin and papain with 2,2'-dipyridyl disulphide and with methyl 2-pyridyl disulphide were determined over a wide range of pH at 25 °C and $I = 0.1$ and the characterizing parameters (pK_a values and pH-independent rate constants) were compared. Although the reactivities of actinidin towards the two probe reagents in each of the three reactive protonic states are similar, the reactivity of papain towards the smaller probe reagent (methyl 2-pyridyl disulphide) is considerably greater than its reactivity towards 2,2'-dipyridyl disulphide. The lower sensitivity of actinidin in its response to structural change in these small catalytic site-specific probe reagents is discussed in terms of a more rigid local conformation. Additional evidence that the reactivity of the catalytic-site thiol group of papain is influenced by additional protonic dissociation with pK_a approx. 4, whereas that of actinidin is not, is provided by the analysis of the pH- k profiles. Implications of the differences in behaviour found for actinidin and papain, which are not readily predicted from existing X-ray crystallographic data, for the study of enzyme mutants produced by site-directed mutagenesis are discussed.

INTRODUCTION

Over the last 20 years there have been two revolutionary advances in the study of enzyme structure and function, namely the development of protein crystallo-

graphy in the 1960s and the introduction of protein engineering by site-directed mutagenesis in the 1980s (see Blow *et al.*, 1986, Leatherbarrow & Fersht, 1986). An approach complementary to the latter, which is particularly useful at the present time while the range of

Abbreviation used: L-Bz-Arg-Nan, *N*^α-benzoyl-L-arginine *p*-nitroanilide.

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functional consequences of structural change by site-directed mutagenesis is being defined, is the study of natural structural variants within a given family of homologous or analogous enzymes (see e.g. Wetzel, 1986). Classical examples of the use of this type of structural variation are to be found in studies on the cytochromes *c* (see e.g., Ferguson-Miller *et al.*, 1979) and on the haemoglobins (see e.g. Perutz, 1983) and natural structural variants continue to be discovered, e.g. in the insulins of some diabetics (Nakagawa & Tager, 1986) and in many oncogenes (Levy *et al.*, 1986). The cysteine proteinases are proving to constitute a particularly valuable enzyme family for assessing the effects of structural variation in the protein on active-centre chemistry and catalytic mechanism (Brocklehurst, 1986, 1987; Brocklehurst *et al.*, 1987b). The particular value of this family of enzymes derives from (a) the existence in some cases, within the family, of considerable structural variation in and around the catalytic site despite, in general, considerable sequence homology (Kamphuis *et al.*, 1985) and (b) the possibility of studying the kinetics of single-step covalency changes related to a crucial stage of the catalytic act by using site-specific reactivity probes. One of the most sensitive, direct, and relevant ways of investigating catalytic-site geometry and its modulation by ligand binding is the study of chemical reactivity characteristics. Transition-state geometry is the fundamental characteristic on which catalytic competence depends and the reactivity of a catalytic-site nucleophile that plays a central role in the catalytic act is a prime target for investigation of factors that help to determine transition-state geometry. In the cysteine proteinases the catalytic-site nucleophile is the thiolate anion of the common cysteine-histidine interactive system. The kinetics and thermodynamics of the processes that produce and destroy this interactive system and provide for covalent bond formation between a ligand and the thiolate anion component can be studied by using particular site-specific electrophilic reagents that discriminate in favour of ion-pair forms as against thiolate anion forms that lack the cationic imidazolium partner. Such discrimination is achieved by using as reactivity probes either reagents that increase their reactivity by protonation at low pH, such as short-chain 2-pyridyl disulphides (two-protonic-state electrophiles; Brocklehurst, 1974, 1982), or reagents such as benzofuroxan (see Salih & Brocklehurst, 1983; Willenbrock & Brocklehurst, 1986) that demand the presence of both $-S^-$ and $-ImH^+$ arranged in a particular geometry for efficient reaction. The value of such reactivity probes and of natural structural variation in cysteine proteinase catalytic sites is illustrated by studies on papain (EC 3.4.22.2) and cathepsins B (EC 3.4.22.1) and H (Willenbrock & Brocklehurst, 1984, 1985a,b, 1986). The residue adjacent on the *N*-terminal side of the catalytic-site histidine residue, which is aspartic acid (Asp-158) in papain, is glycine (Gly-196) in cathepsin B and asparagine (Asn-165) in cathepsin H. These would be the two most obvious changes to make by site-directed mutagenesis in the catalytic site of papain to investigate postulated roles of Asp-158 (see e.g. Brocklehurst *et al.*, 1981). The change Asp $\rightarrow$ Asn preserves the possibility of contacts by hydrogen-bonding while eliminating the negative charge, and the change Asp $\rightarrow$ Gly eliminates all possibilities of significant contacts either with other catalytic site components or with substrate or reactivity probe. A

combination of reactivity probe kinetics and substrate catalysis kinetics suggests that catalytic activity in papain may be produced essentially synchronously with the production of a S^-/ImH^+ ion-pair from SH/ImH^+ perhaps because the ion-pair is subject to the influence of another, positively co-operative, ionization of similar pK_a , possibly associated with the carboxy group of Asp-158. The situations in cathepsins B and H are quite different, both from papain and from each other (Willenbrock & Brocklehurst, 1984, 1985a,b, 1986). No catalytic activity is generated in cathepsin B (where Asp-158 $\rightarrow$ Gly-196), when S^-/ImH^+ is produced by protonic dissociation of pK_a 3.4. Catalytic activity is generated only when another protonic dissociation of pK_a 5–6 occurs. The numerical value of this pK_a suggests a carboxy group in a hydrophobic environment, which is probably remote from the immediate catalytic site because there is no obvious candidate close to the catalytic site that can be deduced from the amino acid sequence of cathepsin B (Takio *et al.*, 1983), the postulated common folding pattern of the cysteine proteinases (Kamphuis *et al.*, 1985) and the three-dimensional structures of papain (Kamphuis *et al.*, 1984) and actinidin (Baker, 1980). The situation in cathepsin H (where Asp-158 $\rightarrow$ Asn-165) is intermediate between that in papain and that in cathepsin B. In cathepsin H, protonic dissociation with pK_a 5–6 is not obligatory for successful acylation by the substrate of the $-S^-/ImH^+$ ion pair (produced by protonic dissociation with pK_a 2.7) but k_{cat} (presumably k_{+2} , the acylation rate constant) is enhanced 5-fold as the pH is raised across pK_a 6.

The working hypothesis is that (a) in papain the field effect from the carboxylate anion of Asp-158 provides for a catalytic-site geometry that allows the $-S^-/ImH^+$ system to be catalytically competent, (b) replacement of the $-CO_2^-$ by $-H$ (the situation in cathepsin B) requires a different mechanism for control of the $-S^-/ImH^+$ geometry with absolute dependence on the ionization of a remote group and (c) replacement of the $-CO_2^-$ by $-CONH_2$ (the situation in cathepsin H) provides sufficient of a hydrogen-bonding capability to allow some catalytic competence in the ion-pair system, which is enhanced by the same remote ionization mechanism on which cathepsin B depends absolutely.

Protein engineering by site-directed mutagenesis should help to define the extent to which the differences in the three enzymes do in fact derive from the variation in the nature of the amino acid residue adjacent to the catalytic-site histidine residue. It is of course widely recognized that even structural variation of this type will not be without interpretational difficulties until conformational consequences remote from the substitution site are defined in particular cases. One problem that may need to be faced is that subtle changes induced in the protein structure by a given substitution may be crucial for chemical and perhaps catalytic behaviour, even though they may not be readily discerned from X-ray diffraction data. The two closely similar enzymes, actinidin (EC 3.4.22.14) and papain, appear to provide a useful test bed for assessment of variation in chemical properties that are not readily predicted from the crystal structures. The molecular structures of these two enzymes have been compared in detail (Baker, 1981; Kamphuis *et al.*, 1985). Superposition of the co-ordinates shows a remarkable degree of similarity, e.g. when all main chain atoms (C, C, O, N) are considered; the r.m.s. deviation for 638

atoms (76% of the polypeptide chain) is only 0.040 nm (see Baker & Drenth, 1987). When those side chains that are identical are included, the agreement is equally good, indicating that the side-chain positions of identical residues are conserved to the same extent as in the case for the main chain. Residues near the active centre, including those commonly supposed to play major roles in the catalytic processes, also superimpose to a degree that approaches the atomic co-ordinate accuracy of both structures. Thus for 20 residues at or around the catalytic site (with both main chain atoms and identical side-chains included) the r.m.s. deviation is only 0.029 nm for 69% of the atoms (Baker & Drenth, 1987). Despite these close similarities in structure, substantial differences in the characteristics and behaviour of the catalytic sites of actinidin and papain have been observed: (a) the pK_a values that characterize the formation and decomposition of the interactive $-S^-/-ImH^+$ system are more widely separated in actinidin than in papain (Brocklehurst *et al.*, 1981); (b) the electrical properties of the two catalytic-site microenvironments are substantially different as assessed by approximately isosteric neutral and anionic reactivity probes (Brocklehurst *et al.*, 1983) and by a thiol-located spectroscopic reporter group (Brocklehurst *et al.*, 1984); (c) the reactivity of the $-S^-/-ImH^+$ ion pair towards benzofuroxan, a small, rigid electrophilic reagent with stringent requirements for the optimal disposition of $-S^-$ and $-ImH^+$, changes across pK_a 5.5 in the case of actinidin but not in the case of papain.

The aims of the work reported here were to determine (a) whether the difference in the behaviour of actinidin and papain towards benzofuroxan is reflected in the pH-dependence of the acylation process of the catalytic act, as it appears to be in the case of cathepsin B (Willenbrock & Brocklehurst, 1986) and (b) whether the two enzymes discriminate differently in thiol reactivity characteristics between two rather similar reactivity probes methyl 2-pyridyl disulphide ($CH_3-S-S-2-Py$) and 2,2'-dipyridyl disulphide (2-Py-S-S-2-Py) in which the difference lies only in the structure of the simple hydrocarbon side chain and not in either electrostatic character or S_2 -subsite binding ability. The results provide additional evidence for substantial differences in properties between these two enzymes despite the closely similar crystal structures. The difference in the pH-dependent control of acylation may be due to a structural difference remote from the active-centre region, but there are clearly differences within the catalytic-site region itself that are detected by chemical reactivity but not in any obvious way thus far by X-ray diffraction.

MATERIALS AND METHODS

Enzymes

Fully active forms of papain and actinidin, each containing 1 mol of thiol/mol of protein, were prepared by methods involving covalent chromatography (see Brocklehurst *et al.*, 1985) reported previously, for papain by Baines & Brocklehurst (1979) and for actinidin by Brocklehurst *et al.* (1981). Concentrations of enzyme protein were calculated from measurements of A_{280} by using the following values of ϵ_{280} ($M^{-1} \cdot cm^{-1}$): for papain, 5.6×10^4 (see Brocklehurst *et al.*, 1973) and for actinidin, 5.5×10^4 (see Brocklehurst *et al.*, 1979). Concentrations of enzyme active sites were determined by reaction with

2,2'-dipyridyl disulphide at pH 4 (Brocklehurst & Little, 1973) using ϵ_{343} $8.08 \times 10^3 M^{-1} \cdot cm^{-1}$ (Stuchbury *et al.*, 1975) to quantify the amount of pyridine-2-thione released. [In the paper by Willenbrock & Brocklehurst; *Biochem. J.* (1984) 222, 805–814, ϵ_{343} is wrongly printed on p. 807, col. 2, line 13, as ϵ_{338} .]

Substrate and reactivity probes

Catalytic activity was investigated by using *N*^α-benzoyl-L-arginine *p*-nitroanilide (L-Bz-Arg-Nan) (Sigma Chemical Co.) and a continuous spectroscopic assay at 410 nm essentially as described for the ficin-catalysed hydrolysis of DL-Bz-Arg-Nan by Malthouse & Brocklehurst (1976) using $\epsilon_{410} = 8.8 \times 10^3 M^{-1} \cdot cm^{-1}$ (Erlanger *et al.*, 1961) which is essentially pH-independent at pH values above 3 (the pK_a of the *p*-nitroanilinium cation is 1.0; Bascombe & Bell, 1959). When corrections are significant they are made by using $\epsilon_{401} = 8.8 \times 10^3 / (1 + 0.1[H^+]) M^{-1} \cdot cm^{-1}$. The stock solution of L-Bz-Arg-Nan was made up in water (containing 1% v/v dimethyl sulphoxide) rather than in buffer to facilitate pH-dependence studies in which buffer is added separately to the reaction mixtures in the kinetic runs.

2,2'-Dipyridyl disulphide was the Aldrich product, twice recrystallized from light petroleum (b.p. 60–80 °C) and had m.p. 58 °C (Marchwald *et al.*, 1900, give m.p. 57–58 °C). Saturated solutions (approx. 1.5 mM) were prepared by stirring solid reagent in water at room temperature (approx. 22 °C) for 30 min and removing undissolved solid by filtration. Such solutions, stored in stoppered flasks shielded from light with aluminium foil, are stable for at least 1 month. Concentrations were determined by spectral analysis at pH 7 ($\epsilon_{281} = 1.0 \times 10^4 M^{-1} \cdot cm^{-1}$) and were checked occasionally by thiolysis of the 2,2'-dipyridyl disulphide with excess of 2-mercaptoethanol at pH 7 and spectral analysis of the resulting pyridine 2-thione ($\epsilon_{343} = 8.08 \times 10^3 M^{-1} \cdot cm^{-1}$; 1 mol of 2,2'-dipyridyl disulphide provides 2 mol of pyridine 2-thione).

Methyl 2-pyridyl disulphide (first described by Kimura *et al.*, 1982) was synthesized by the thiomethylation of pyridine 2-thione by a procedure similar to that used by Kitson & Loomes (1985). Pyridine 2-thione (3.1 g, 28 mmol) was dissolved in water containing NaOH (1.1 g, 28 mmol) and to this solution methyl methane thiol-sulphonate (Sigma, 3.5 g, 28 mmol) was added at room temperature, approx. 22 °C, with stirring. The mixture immediately became cloudy and white and a pale yellow oil separated out after about 0.5 h. The mixture was allowed to stir for a further 2 h after which it was extracted with methylene chloride (3 × 55 ml). The methylene chloride layer was dried over anhydrous Na_2SO_4 overnight and the solid was then removed by filtration. Removal of the solvent by rotary evaporation *in vacuo* left crude methyl 2-pyridyl disulphide as a yellow oil, which was purified by column chromatography on two silica gel (Kieselgel 60, Merck) columns (33 cm × 1.5 cm). The oil was applied to the first column and toluene/ethyl acetate (4:1, v/v) was used as eluent: 40 ml fractions were collected. Those fractions containing yellow solution were pooled and the solvent was removed by rotary evaporation *in vacuo*. The pale yellow oil was subjected to further chromatography on a fresh column of silica gel and solvent was removed as described above. The product, a pale yellow oil, was stored at 4 °C. A sample produced the predicted yield of pyridine 2-thione

consequent upon thiolysis with 2-mercaptoethanol at pH 7, deduced by spectral analysis at 343 nm ($\epsilon_{343} = 8.08 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$; Stuchbury *et al.*, 1975).

Kinetics

All reactions were studied at 25 °C and I 0.1 in solutions containing 1 mM-EDTA. For stopped-flow runs with the disulphide reactivity probes (Dionex equipment and Tektronix 5103N storage oscilloscope, λ 343 nm; $\Delta\epsilon_{343} = 8.08 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$; Stuchbury *et al.*, 1975) buffer solutions were prepared double-strength such that dilution by 1:2 during reaction in the mixing chamber gave I 0.1, by using the data of Long (1971) and Dawson *et al.* (1969) for the following systems: glycine/HCl (pH 2.0–2.6); formic acid/NaOH (pH 2.8–4.0); acetic acid/NaOH (pH 4.2–5.4); KH_2PO_4 /NaOH (pH 5.8–7.8); Tris/HCl (pH 8.0–8.6); H_3BO_3 /KCl/NaOH (pH 5.8–7.8); Tris/HCl (pH 8.0–8.6); H_3BO_3 /KCl/NaOH (pH 8.2–8.6); $\text{Na}_4\text{P}_2\text{O}_7$ /HCl (pH 8.0–9.4); glycine/NaOH (pH 9.0–10.2); NaHCO_3 /NaOH (pH 9.0–10.6). For substrate hydrolysis kinetics (Cary 118C spectrophotometer) buffers were prepared triple strength such that dilution by 1:3 gave $I = 0.1$.

RESULTS AND DISCUSSION

pH-dependence of the acylation of actinidin by L-Bz-Arg-Nan and comparison with the analogous acylation of papain

L-Bz-Arg-Nan was selected as a substrate with which to investigate the pH-dependent kinetics of actinidin-catalysed hydrolysis because, for the catalysis at pH 6, the values of k_{cat}/K_m and K_m reported by Baker *et al.* (1980) appeared to be appropriate for this type of study. Thus the value of k_{cat}/K_m (reported to be $13 \text{ M}^{-1} \cdot \text{s}^{-1}$ but found to be somewhat lower, $7 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 6, in the present work) is low enough to ensure that (a) the pH-dependence of this apparent second-order rate constant reveals molecular pK_a values characteristic of the free enzyme molecule (Brocklehurst & Dixon, 1977) and (b) K_m provides an excellent approximation to K_s , the dissociation constant of the enzyme–substrate complex (Brocklehurst, 1979), so that k_{cat}/K_m provides k_{+2}/K_s [i.e. the overall second-order rate constant for the acylation process via quasi-equilibrium binding of enzyme (E) and substrate (S)]. In addition, the value of K_m (approx. 2 mM) provides the opportunity to evaluate k_{cat}/K_m from single kinetic runs at each pH with $[S] \approx 0.1 \text{ mM}$. At this concentration initial rates are readily measured by using the 0–0.2 range of a Cary 118C spectrophotometer. Complete hydrolysis of 0.1 mM-L-Bz-Arg-Nan in a 1 cm path length cell provides $\Delta A_{410} = 0.88$. The same considerations make L-Bz-Arg-Nan suitable for an analogous study of papain-catalysed hydrolysis for which data from other laboratories already exist (see Lewis *et al.*, 1978).

In the present work the actinidin-catalysed hydrolysis of L-Bz-Arg-Nan was shown to adhere well to Michaelis–Menten kinetics at pH values 4.2, 7.1 and 9.5 by the linearity of plots of $[S]_0/v$ versus $[S]_0$ (not shown) for values of $[S]_0$ between 0.2 mM and 6.4 mM. The K_m values at these pH values (2.6 mM at pH 4.2, 1.8 mM at pH 7.1 and 11.2 mM at pH 9.5) confirmed that good estimates of k_{cat}/K_m (and hence of k_{+2}/K_s) should be obtained over a range of pH by dividing v by $[E]_T$ (the total concentration of active enzyme) and $[S]_0$.

Fig. 1. pH-dependence of $k_{\text{cat}}/K_m (= k_{+2}/K_s)$ for the hydrolysis of L-Bz-Arg-Nan catalysed by actinidin at 25 °C and $I = 0.1$

The points are experimental for reactions with $[E]_T = 1.0 \mu\text{M}$ and $[S]_0 = 0.11 \text{ mM}$; values of k_{cat}/K_m were calculated from $k_{\text{cat}}/K_m = v/[E]_T[S]_0$. Values of k_{cat}/K_m similar to those shown were obtained at pH values of around 4, 7 and 9 from values of k_{cat} and K_m separately determined. The solid line is theoretical for:

$$\frac{k_{\text{cat}}}{K_m} = \frac{\tilde{k}_{\text{cat}}/\tilde{K}_m}{1 + \frac{[\text{H}^+]}{K_I} + \frac{K_{II}}{[\text{H}^+]} + \frac{K_I K_{II}}{[\text{H}^+]^2}} + \frac{\tilde{k}'_{\text{cat}}/\tilde{K}'_m}{1 + \frac{[\text{H}^+]^2}{K_I K_{II}} + \frac{[\text{H}^+]}{K_{II}} + \frac{K_{III}}{[\text{H}^+]}}$$

where $\text{p}K_I = 3.1$, $\text{p}K_{II} = 5.5$, $\text{p}K_{III} = 9.7$, $\tilde{k}_{\text{cat}}/\tilde{K}_m = 2.75 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $\tilde{k}'_{\text{cat}}/\tilde{K}'_m = 8.5 \text{ M}^{-1}$. The broken line is theoretical for:

$$\frac{k_{\text{cat}}}{K_m} = \frac{\tilde{k}_{\text{cat}}/\tilde{K}_m}{1 + \frac{[\text{H}^+]}{K}}$$

where $\text{p}K = 5.5$ and $\tilde{k}_{\text{cat}}/\tilde{K}_m = 8.5 \text{ M}^{-1} \cdot \text{s}^{-1}$.

The pH-dependence of k_{+2}/K_s thus determined is shown in Fig. 1. The data in the pH range 5.5–10 conform reasonably well to a conventional bell-shaped curve (with $\text{p}K_I = 5.5$ and $\tilde{k}_{+2}/\tilde{K}_s = 8.5 \text{ M}^{-1} \cdot \text{s}^{-1}$) but at lower pH values the data convincingly lie above the acid limb of the bell. The solid line in Fig. 1, which fits the complete data set collected in the pH range 3.8–10, corresponds to eqn. (1) with $\text{p}K_{eI} = 3.1$, $\text{p}K_{eII} = 5.5$, $\text{p}K_{eIII} = 9.7$, $\tilde{k}_{+2}/\tilde{K}_s = 2.75 \text{ M}^{-1} \cdot \text{s}^{-1}$, and $\tilde{k}'_{+2}/\tilde{K}'_s = 8.5 \text{ M}^{-1} \cdot \text{s}^{-1}$.

$$\frac{k_{+2}}{K_s} = \frac{\tilde{k}_{+2}/\tilde{K}_s}{1 + \frac{[\text{H}^+]}{K_{eI}} + \frac{K_{eII}}{[\text{H}^+]} + \frac{K_{eI} K_{eII}}{[\text{H}^+]^2}} + \frac{\tilde{k}'_{+2}/\tilde{K}'_s}{1 + \frac{[\text{H}^+]^2}{K_{eI} K_{eII}} + \frac{[\text{H}^+]}{K_{eII}} + \frac{K_{eIII}}{[\text{H}^+]}} \quad (1)$$

Scheme 1. Kinetic model for the acylation of actinidin (E) by L-Bz-Arg-Nan (S) in two active ionization states

EH₃S and EHS are adsorptive complexes that lead to the production of acyl enzyme intermediates EH₂S' and EHS' respectively with concomitant formation of *p*-nitroaniline (P₁). When acylation is rate-determining, as in the case of the catalysed hydrolysis of L-Bz-Arg-Nan, the regeneration of enzyme forms from the acyl enzyme intermediates (not shown) is rapid.

This equation describes the pH-dependence of k_{+2}/K_s in terms of the model shown in Scheme 1 and is a particular example of the general equation discussed by Brocklehurst (1974). This model provides for acylation of actinidin to take place in two ionization states, that of lower reactivity being produced by protonic dissociation in the free enzyme molecule characterized by $\text{p}K_a < 4$, and that of substantially higher reactivity being produced by protonic dissociation across a free enzyme $\text{p}K_a$ of 5.5. It seems reasonable to postulate that the $\text{p}K_a < 4$ might characterize the formation of the state of the enzyme that contains the $-\text{S}^-/\text{ImH}^+$ catalytic-site ion pair, by protonic dissociation from $-\text{SH}/\text{ImH}^+$. This process is characterized by $\text{p}K_a$ 3.1 as deduced from two-protonic-state reactivity probe kinetics (see below) and it is difficult to envisage an acylation mechanism that does not involve an enzyme form containing nucleophilic sulphur. It was not possible to study the kinetics of the actinidin-catalysed hydrolysis at pH values significantly lower than 4 because of loss of the conformational integrity of the catalytic site. This was detected by titration of intact actinidin catalytic sites with 2,2'-dipyridyl disulphide using a method based on that devised for titration of papain catalytic sites (Brocklehurst & Little, 1973) which has general applicability for many cysteine proteinases (Brocklehurst, 1982). It is the folding of the protein that provides for the proximity of catalytic-site thiol and imidazole groups and hence the interactive system containing the nucleophilic $-\text{S}^-/\text{ImH}^+$ ion pair. The co-existence in acidic media of significant concentrations of this ion pair and the highly reactive 2,2'-dipyridyl disulphide monocation results in a rapid release of pyridine 2-thione, which is monitored at 343 nm. Incubation of the enzyme (3.5 μM) at a particular pH for an appropriate time followed by spectral titration with 2,2'-dipyridyl disulphide (140 μM) at pH 4, defines the pH range over which all of the enzyme molecules retain functional catalytic sites over the incubation period. Two incubation periods were evaluated, one of 3–5 min which is appropriate for initial rate measurements of L-Bz-Arg-Nan hydrolysis and the other of 1–2 s which is appropriate for the stopped-flow kinetic

studies with disulphide reactivity probes (discussed below). At pH values where no conformational distortion of the catalytic site occurs over a given incubation time, reaction with 2,2'-dipyridyl disulphide at pH 4 provides a yield of pyridine 2-thione stoichiometric with the catalytic site (determined by reaction with 2,2'-dipyridyl disulphide at pH 6 where the enzyme is conformationally stable). Conformational distortion over the incubation period provides a yield of pyridine 2-thione that is less than stoichiometric in the rapid phase of reaction. Subsequent reaction of conformationally distorted enzyme with 2,2'-dipyridyl disulphide does not occur at a significant rate at $\text{pH} \leq 4$ over the stopped-flow time scale.

Over the initial rate time scale (3–5 min incubation) actinidin is conformationally stable in acid media at pH values > 4 . At pH 3.5, 90% of the catalytic sites survive the incubation; at pH 3.35, 64% survive, and at pH 3.2, the native catalytic-site conformation is totally lost. Over the stopped-flow time scale (1–2 s incubation) actinidin is conformationally stable in acidic media at pH values > 2.5 . At pH 2.4, 75% of the catalytic sites survive the incubation, and at pH 2.1, 40% survive. The data in Fig. 1 provide evidence that the existence of $-\text{S}^-/\text{ImH}^+$ ion pair in the actinidin molecule is not a sufficient condition for the expression of full reactivity in the acylation process and that an additional protonic dissociation ($\text{p}K_a$ 5.5 in the free enzyme) results in a 3-fold increase in overall reactivity (from $2.75 \text{ M}^{-1} \cdot \text{s}^{-1}$ to $8.5 \text{ M}^{-1} \cdot \text{s}^{-1}$). This type of enhancement was postulated on the basis of the pH-dependence of the rate of reaction of actinidin towards benzofuroxan and by analogy with our studies of the acylation processes in hydrolyses catalysed by cathepsins B and H (see the Introduction). Actinidin appears to resemble cathepsin H in that acylation of the enzyme can occur, albeit at a relatively low rate, without protonic dissociation of $\text{p}K_a$ 5–6 and cathepsin B is still unique in exhibiting an absolute requirement for two distinct, widely separated, ionizations in acidic media to produce catalytic competence.

The similarity of actinidin to cathepsin H, and to some extent to cathepsin B, is in marked contrast to the characteristics that have been reported for the acylation of papain by a number of substrates including L-Bz-Arg-Nan (see Lewis *et al.*, 1978 and references cited therein). For papain-catalysed hydrolysis, the increase in k_{cat}/K_m (and hence k_{+2}/K_s) in acidic media was reported to depend on the protonic dissociation of two groups with $\text{p}K_a$ values close to 4. Because this conclusion depends heavily on kinetic measurements at low pH, we re-investigated the pH-dependence of k_{cat}/K_m for the papain-catalysed hydrolysis of L-Bz-Arg-Nan and included corrections for loss of catalytic-site geometry in acidic media calculated from the results of titrations using 2,2'-dipyridyl disulphide analogous to those described above for actinidin.

Over the stopped-flow time scale (1–2 s incubation) the acid stability characteristics of papain (3.5 μM) are closely similar to those reported above for actinidin. Over the initial rate time scale (3–5 min incubation), papain is somewhat more resistant than actinidin to acid-promoted denaturation: for example, papain is conformationally stable in acidic media at pH values > 3.2 and 60% of the catalytic sites survive at pH 2.9.

The pH-dependence of k_{cat}/K_m (k_{+2}/K_s) for the papain-catalysed hydrolysis of L-Bz-Arg-Nan in acidic

Fig. 2. pH-dependence of $k_{\text{cat}}/K_m (= k_{+2}/K_2)$ for the hydrolysis of L-Bz-Arg-Nan catalysed by papain in the acidic media at 25 °C and $I = 0.1$

The points are experimental for reactions with $[E]_T = 1.3 \mu\text{M}$ and $[S]_0 = 0.1 \text{ mM}$; values of k_{cat}/K_m were calculated as indicated for the data shown in Fig. 1. The solid line is theoretical for:

$$\frac{k_{\text{cat}}}{K_m} = \frac{\tilde{k}_{\text{cat}}/\tilde{K}_m}{1 + \frac{[H^+]}{K}}, \text{ where } pK = 4.1 \text{ and } \tilde{k}_{\text{cat}}/\tilde{K}_m = 206 \text{ M}^{-1} \cdot \text{s}^{-1}.$$

The value of $[E]_T$ is that determined by titration of catalytic sites by 2,2'-dipyridyl disulphide at pH values of 4, where the enzyme is conformationally stable.

media, determined from initial rate measurements with $[S]_0 \ll K_m$ and using the value of $[E]_T$ calculated by titration with 2,2'-dipyridyl disulphide at pH 4, is shown in Fig. 2. At pH values above 3.5 the acylation of papain is characterized by a sigmoid pH-dependence curve with $pK_1 = 4.1$, and $\tilde{k}_{+2}/\tilde{K}_s = 206 \text{ M}^{-1} \cdot \text{s}^{-1}$, which is similar to that ($201 \text{ M}^{-1} \cdot \text{s}^{-1}$) reported by Lewis *et al.* (1978). The data in Fig. 2 at pH values below 3.5 fall consistently below the fitted curve and this failure to fit to an acid limb characterized by a single pK_a value of around 4 is maintained when the loss of catalytic-site geometry determined by titration with 2,2'-dipyridyl disulphide is allowed for (Fig. 3). The present study, therefore, which includes correction for loss of catalytic site conformation, supports the conclusions reached by earlier studies that the acylation of papain depends upon two protonic dissociations in the papain molecule with pK_a values approx. in the range 3–4.

It is also clear from Fig. 2 that a protonic dissociation with a pK_a in the region 5–6 is not a major influence on the development of catalytic activity in papain as it is in actinidin (Fig. 1). It seems interesting that a pK_a of 5–6 controls both the thiol reactivity of actinidin and of cathepsin B towards benzofuroxan (Salih & Brockle-

Fig. 3. pH-dependence of $k_{\text{cat}}/K_m (= k_{+2}/K_2)$ for the hydrolysis of L-Bz-Arg-Nan catalysed by papain in the pH range 2.7–3.3

The points denoted by ● are the data points from Fig. 2; those denoted by ■ show the effect on the values of k_{cat}/K_m of correcting the enzyme concentration for loss of conformational integrity at pH values of < 3 by titrating intact catalytic sites with 2,2'-dipyridyl disulphide at the same pH values as those used to determine the values of k_{cat}/K_m . Line (a) is theoretical for the equation given in Fig. 2 legend where, as in Fig. 2, $pK = 4.1$ and $\tilde{k}_{\text{cat}}/\tilde{K}_m = 206 \text{ M}^{-1} \cdot \text{s}^{-1}$. Lines (b)–(d) are theoretical for:

$$\frac{k_{\text{cat}}}{K_m} = \frac{\tilde{k}_{\text{cat}}/\tilde{K}_m}{1 + \frac{[H^+]}{K_1} + \frac{K_1}{[H^+]}} + \frac{\tilde{k}'_{\text{cat}}/\tilde{K}'_m}{1 + \frac{[H^+]^2}{K_1 K_{II}} + \frac{[H^+]}{K_{II}}}$$

where, in all cases, $\tilde{k}_{\text{cat}}/\tilde{K}_m = 206 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $pK_{II} = 4.1$, and the values of pK_1 and $\tilde{k}'_{\text{cat}}/\tilde{K}'_m$ are respectively: for (b) 3.7 and $40 \text{ M}^{-1} \cdot \text{s}^{-1}$, for (c) 3.3 and $10 \text{ M}^{-1} \cdot \text{s}^{-1}$, and for (d) 3.0 and $2 \text{ M}^{-1} \cdot \text{s}^{-1}$.

hurst, 1983; Willenbrock & Brocklehurst, 1986) and the catalytic activity (acylation) of both enzymes, whereas in the case of papain, neither the thiol reactivity towards benzofuroxan (Shipton & Brocklehurst, 1977) nor the catalytic activity is modulated by protonic dissociation with pK_a 5–6. Benzofuroxan is a small rigid electrophilic reagent that might be expected to require a precise geometry for the disposition of $-S^-$ and $-ImH^+$ components of ion-pairs for optimal concerted attack (see Willenbrock & Brocklehurst, 1986). If the dependence on pK_a 5–6 of both the benzofuroxan reaction and the acylation process in catalysis have common cause, therefore, control of ion-pair geometry by protonic dis-

sociation with pK_a 5–6 might be the explanation for the dependence of k_{cat}/K_m on this pK_a .

One question that remains to be answered is why the pK_a of 5–6 is an important feature of actinidin catalytic-site chemistry and catalytic character but not of the analogous qualities in papain. This is an intriguing question in view of the very close similarities in structure in and around the two enzyme catalytic sites deduced by X-ray crystallographic study (see the Introduction). One possibility of course is that the group with pK_a 5.5 in actinidin is remote from the catalytic-site region, where structural differences between actinidin and papain are known to exist (Kamphuis *et al.*, 1985; Baker & Drenth, 1987). In cathepsin B, the functionally analogous group is probably remote from the catalytic site because the amino acid residue analogous in the sequence to Asp-158

provided by the reactivity probe kinetics discussed below.

Investigation of the catalytic site regions of actinidin and papain by two-protonic-state reactivity probes

Objectives and strategy. This part of the investigation was designed to compare the responses of the two enzymes to structural change in small, thiol-specific probe reagents where enzyme–reagent contacts should be limited to the region in and close to the catalytic site. In this region the structures of the two enzymes deduced by X-ray crystallography appear to be closely similar (see the Introduction). Reactivities of the two enzymes towards 2,2'-dipyridyl disulphide (I) were compared with each other and with those for the corresponding reactions with methyl 2-pyridyl disulphide (II).

of papain is Gly in cathepsin B (Takio *et al.*, 1983) and there is no obvious substitute for such a group that can be deduced from the amino acid sequence of cathepsin B, the postulated common folding pattern of the cysteine proteinases and the three-dimensional structures of papain and actinidin.

It must remain a possibility that the catalytically relevant pK_a close to 4 in papain additional to that assigned to the protonic dissociation of the catalytic site $-SH/-ImH^+$ system may too characterize a group remote from the catalytic-site region, although a favoured hypothesis has long been that it might be due to Asp-158. The fact that a catalytic site (thiol)-located nitrobenzofurazan spectroscopic label detects a group in papain ionizing with pK_a 3.7 but detects no ionizing group with a pK_a in this region in actinidin (Brocklehurst *et al.*, 1984) tends to suggest that Asp-158 in papain and the analogous aspartic acid residue in actinidin may not be conformationally equivalent in solution, despite the close similarity in the crystal. Furthermore, the fact that the spectroscopic label attached to the catalytic-site sulphur atom of actinidin is also not affected by pH change around 5.5 (indeed up to pH 8) argues against functionally equivalent, remote ionizing groups in papain and actinidin, with that in papain having pK_a approx. 4 and that in actinidin having pK_a 5.5. The weight of evidence discussed here and in the Introduction seems to favour a papain catalytic site affected substantially by the ionization of Asp-158 and an actinidin catalytic site affected instead by ionization of a remote group with pK_a 5.5.

Additional evidence for differences between actinidin and papain in the immediate catalytic site region is

Both reagents are two-protonic-state electrophiles (Brocklehurst, 1982) and permit the reactivities of the enzyme catalytic-site thiol groups to be studied over a wide range of pH so that reactivities of both the interactive $-S^-/-ImH^+$ systems and the uncomplicated $-S^-/-Im$ thiolate anions can be determined.

Conformational changes in the enzymes in acidic media.

The possibility of using 2,2'-dipyridyl disulphide to titrate the concentration of non-distorted catalytic sites in acidic media was discussed above in connection with the study of catalytic activity. One objective of pH-dependent kinetic studies with reactivity probes is to determine the pK_a characteristic of the formation of catalytic site $-S^-/-ImH^+$ ion pair and the reactivity of this enzyme state with the cationic probe. This requires knowledge of the conformational behaviour of the enzymes in acidic media to determine the low pH limit of conformational stability above which valid kinetic data may be collected. When the enzyme catalytic site reacts with the pyridyl disulphide to release pyridine 2-thione in a single- (pseudo-first-order) phase progress curve in an amount stoichiometric with enzyme catalytic sites, there is no evidence for the occurrence of acid-promoted conformational distortion within the time-scale of a stopped-flow experiment. Concentrations of native catalytic sites are determined by titration with 2,2'-dipyridyl disulphide at pH 4–6 where the enzymes are conformationally stable.

The simplest model for loss of stoichiometry of the thiol-modification reaction in acidic media with a particular concentration of reagent is eqn. (2) in which, within the time-scale of a stopped-flow run, only a proportion of the catalytic sites survives in the conform-

ation necessary to maintain ion pair and thus to react rapidly with 2,2'-dipyridyl disulphide (R) to produce E-S-S-2-Py and the chromophoric pyridine 2-thione (P). The rest undergo conformational change to E', in which the ion-pair geometry is perturbed with consequent protonation of the thiolate ion component and hence loss of high reactivity towards R. When considering the loss of conformational integrity in terms of both eqn. (1) and eqn. (2) nothing is gained by complicating the models by the introduction of adsorptive ER complexes.

If this model could be shown to describe the behaviour of these enzymes in acidic media, it would be possible to treat the kinetic data (with $[\text{R}] \gg [\text{E}]$) as a pair of competing first-order reactions and thus evaluate k (and k') at lower pH values than those providing for stoichiometric release of P. In the model of eqn. (2), E partitions within the stopped-flow time scale by unimolecular conformational change which does not produce P, and bimolecular reaction with R, which does produce P. Thus the model predicts that by increasing $[\text{R}]$, stoichiometric release of P should be achieved or approached, i.e. $[\text{P}]_{\infty}/[\text{E}]_0$ should tend to 1.0 as $[\text{R}]$ is increased. The data in Fig. 4 demonstrate that the behaviour predicted by this model does not occur with either actinidin or papain. Instead, although there is some increase in $[\text{P}]_{\infty}/[\text{E}]_0$ with increase in $[\text{R}]$, the limiting value, $([\text{P}]_{\infty}/[\text{E}]_0)_{\text{lim}}$, decreases with decreasing pH and at the lower pH values is $\ll 1$. Fig. 5 shows how $([\text{P}]_{\infty}/[\text{E}]_0)_{\text{lim}}$ varies with pH. For papain, conformational integrity is maintained down to about pH 2.8. Then product stoichiometry decreases with decreasing pH until another plateau region is reached at around pH 1.5. With actinidin, loss of conformational integrity begins at a somewhat higher pH (about pH 3.3). The decrease in product stoichiometry with decrease in pH is similar to the situation found with papain down to about pH 2.1 but then a sharp transition occurs leading to more sensitive change of conformation.

The results presented in Figs. 4 and 5 require a model like that shown in eqn. (3) in which the bimolecular reaction with R is separated from the process leading to loss of conformational integrity by a potentially reversible step.

When E is introduced to the low-pH buffer for a few seconds before the stopped-flow experiment in which enzyme and R are brought together is carried out, $[\text{P}]_{\infty}/[\text{E}]_0 = 0$, in accord with eqn. (3) when E is converted completely to E'. As $[\text{R}]$ is increased, $[\text{P}]_{\infty}/[\text{E}]_0$ increases until $k_{+2}[\text{R}] \gg k_{-1}$, when $\text{E}^* + \text{R} \rightarrow \text{P}$ becomes insulated from $\text{E} \rightarrow \text{E}'$ and no further increase in $[\text{P}]_{\infty}/[\text{E}]_0$ is possible. Even under these conditions $k_{+2}[\text{R}] \ll k_{+1}$ because the observed first-order rate constant for production of P increases linearly with increasing $[\text{R}]$.

Fig. 4. Dependence of the yield on the stopped-flow time scale of pyridine 2-thione (P) in acidic media on the concentration of 2,2'-dipyridyl disulphide in its reaction with (a) papain (2 μM) and (b) actinidin (2 μM)

The reactions were carried out at 25 °C and $I = 0.1$; $[\text{P}]_{\infty}$ was calculated from the amplitude of the single-phase stopped-flow record at 343 nm and $\Delta\epsilon_{343} = 8080 \text{ M}^{-1} \cdot \text{cm}^{-1}$; $[\text{E}]_0$ was calculated in a similar way from a reaction carried out at pH 6 where the amplitude is independent of the concentration of 2,2'-dipyridyl disulphide provided that it is at least equal to the concentration of catalytic sites.

Fig. 5. pH-dependence of $([P]_{\infty}/[E]_0)_{lim}$ for the reactions of 2,2'-dipyridyl disulphide with (a) papain and (b) actinidin

Values of $([P]_{\infty}/[E]_0)_{lim}$ are the limiting values of $[P]_{\infty}/[E]_0$ in Fig. 4 obtained by inspection of the data at high concentrations of 2,2'-dipyridyl disulphide where the values are essentially independent of the concentration of 2,2'-dipyridyl disulphide.

The value of $([P]_{\infty}/[E]_0)_{lim}$ at a given pH where eqn. (3) applies is determined by the relative values of k_{+1} and k'_{+1} . The fact that for papain, the values of the second-order rate constants (k) for the reaction with 2,2'-dipyridyl disulphide at pH values below 2.7 are those predicted from the pH-dependence at higher pH where conformational integrity is maintained (Fig. 6), suggests that the catalytic-site geometry in E^* is not substantially

Fig. 6. pH-dependence of the second-order rate constant (k) for the reaction of papain with (a) methyl 2-pyridyl disulphide and (b) 2,2'-dipyridyl disulphide in aqueous media at 25 °C and $I = 0.1$

The points are experimental and the lines are theoretical for:

$$\frac{\tilde{k}_1}{1 + \frac{[H^+]}{K_I} + \frac{K_{II}}{[H^+]}} + \frac{\tilde{k}_2}{1 + \frac{[H^+]^2}{K_I K_{II}} + \frac{[H^+]}{K_{II}} + \frac{K_{III}}{[H^+]}} + \frac{\tilde{k}_3}{1 + \frac{[H^+]}{K_{III}}}$$

(see Shipton & Brocklehurst, 1978) and the following are the values of the parameters, those for (a) being given first in each case and those for (b) being in parentheses: $pK_I = 3.9$ (3.85); $pK_{II} = 4.0$ (3.9); $pK_{III} = 9.5$ (9.5); $\tilde{k}_1 = 2.25 \times 10^5$ (4.2×10^4) $M^{-1} \cdot s^{-1}$; $\tilde{k}_2 = 2.2 \times 10^4$ (7.0×10^2) $M^{-1} \cdot s^{-1}$; $\tilde{k}_3 = 3.9 \times 10^4$ (1.7×10^3) $M^{-1} \cdot s^{-1}$. For (b) the values of pK_I , pK_{II} and $\tilde{k}_1$ are those reported by Shipton & Brocklehurst (1978) and the fitted curve describes the main features of the data in acidic media (with the data points around pH 5 lying above the fitted line). The arrows indicate the pH range over which data were collected by Shipton & Brocklehurst (1978). In the present work a more extensive data set was collected in alkaline media which permitted a better characterization of the curve in the range pH 7–10.5.

different from that in E. The same conclusion applies to actinidin in the pH range of about 2.1–3.2 but the sharp transition in Fig. 5(b) at about pH 2.1 is reflected in the kinetics of the reaction of actinidin with 2,2'-dipyridyl disulphide. At pH values below about 2.1, the values of

Fig. 7. pH-dependence of the second-order rate constant for the reaction of actinidin with (a) methyl 2-pyridyl disulphide and (b) 2,2'-dipyridyl disulphide in aqueous media at 25 °C and $I = 0.1$

The points are experimental and the lines are theoretical for the equation given in Fig. 6 legend and the following are the values of the parameters, those for (a) being given first in each case and those for (b) being in parentheses: $pK_I = 2.8$ (2.5); $pK_{II} = 3.5$ (3.0); $pK_{III} = 10.0$ (10.0); $k_1 = 2.9 \times 10^4$ (2.4×10^4) $M^{-1} \cdot s^{-1}$; $k_2 = 8.5 \times 10^2$ (1.9×10^3) $M^{-1} \cdot s^{-1}$; $k_3 = 4.0 \times 10^3$ (5.2×10^3) $M^{-1} \cdot s^{-1}$.

k (not shown) are much greater than would be predicted from the behaviour at higher pH values (Fig. 7), which suggests that the first-order rate constants from which values of k are calculated contain an additional component which may be related to a conformational transition in E^* [of eqn. (3)] leading to loss of rapid reaction with R.

pH-dependent kinetic studies. The pH-dependences of the second-order rate constants (k) for the reactions of 2,2'-dipyridyl disulphide and of methyl 2-pyridyl disulphide each with the catalytic-site thiol groups of papain and actinidin are shown in Figs. 6 and 7. All reactions were carried out under pseudo-first-order conditions with the disulphide probe reagent in considerable excess over the enzyme and the adherence to overall second-order kinetics was established for all reactions by the linearity of plots of pseudo-first-order rate constant against [disulphide] at around pH 4, 6 and 8. The reactivities of the two enzymes towards both probe reagents in the three reactive protonic states are compared in Table 1.

There are two marked differences between the behaviour of the two enzymes. One is that the bell-shaped components in the pH- k profiles for the papain reactions are characterized by two enzyme pK_a values (both approx. 4) whereas the analogous components for the corresponding actinidin reactions are characterized by one enzyme pK_a and the reagent pK_a , determined by spectral analysis (Fig. 8). The existence of co-operativity involving two ionizing groups in papain with pK_a ca. 4 has been discussed previously (Brocklehurst *et al.*, 1981). The other marked difference concerns the reactivities shown in Table 1. The small change in the structure of the probe reagent has very little effect on its reactivity towards actinidin in any of the three reactive protonic states. In marked contrast to this situation, the replacement of 2-pyridyl- by methyl- in the probe results in a substantial increase in reactivity towards papain. This increase is all the more remarkable because the intrinsic electrophilic reactivity of alkyl 2-pyridyl disulphides towards simple low- M_r mercaptans is considerably lower (often 5–10 fold) than the corresponding reactivity of 2,2'-dipyridyl disulphide (T. Stuchbury & K. Brocklehurst, unpublished work). The most obvious explanation for the lower observed reactivity of 2,2'-dipyridyl disulphide towards papain is that the presence of the additional pyridyl ring decreases the binding ability of the reagent in the catalytic-site region. The lower sensitivity of actinidin in its response to this type of structural change in the probe would not have been readily predicted from the crystallographic data in view of the superimposable protein structures in the catalytic-site region. It may correlate, however, with a difference between the two enzymes detected by 2-(acetamido)ethyl 2'-pyridyl disulphide (III) and by the substrate analogue 2-(N' -acetyl-L-phenylalanyl-amino)ethyl 2'-pyridyl disulphide (IV) (Brocklehurst *et al.*, 1987a).

The marked difference in behaviour between the two enzymes towards these probes appears to involve in part a failure of actinidin in the interaction of the P_1 - P_2 amide bond across the active centre cleft to transmit the hydrophobic binding interaction in the S_2 subsite.

In the present work, the insensitivity of actinidin to replacement of one pyridyl group in the probe by methyl, a structural change devoid of complications arising from specific hydrogen-bonding interactions like those predicted with (III) and (IV), appears to constitute evidence for a more rigid structure in the S_1 - S_2 region of actinidin than in the corresponding region of papain. This difference in rigidity could be an important factor in

Table 1. Characteristics of the reactions at 25 °C and $I = 0.1$ of the catalytic-site thiol groups of actinidin and papain with 2,2'-dipyridyl disulphide (2-Py-S-S-2-Py, 2PDS) and with methyl 2-pyridyl disulphide ($\text{CH}_3\text{-S-S-2-Py}$, MPDS) in the three main reactive protonic states, designated X, XH, and XH_2 to indicate their relative stoichiometry in protons

Thiol group location	Probe	$\text{p}K_{\text{I}}$	$\text{p}K_{\text{II}}$	$\text{p}K_{\text{III}}$	pH-independent rate constants ($\text{M}^{-1}\cdot\text{s}^{-1}$)			$\tilde{k}_{\text{MPDS}}/\tilde{k}_{\text{2PDS}}$		
					$\tilde{k}_{\text{XH}_2}$	$\tilde{k}_{\text{XH}}$	$\tilde{k}_{\text{X}}$	XH_2	XH	X
Actinidin	$\text{CH}_3\text{-S-S-2-Py}$	2.8	3.5	10.0	29 000	850	4000	1.2	0.45	0.8
	2-Py-S-S-2-Py	2.8	3.0	10.0	24 000	1900	5200			
Papain	$\text{CH}_3\text{-S-S-2-Py}$	3.9	4.0	9.6	225 000	22 000	39 000	5.4*	31	23
	2-Py-S-S-2-Py	3.85	3.9	9.5	42 000	700	1700			

* For the papain reactions which display positive co-operativity in the protonic dissociations around the XH_2 state, very small errors in the $\text{p}K_{\text{a}}$ values result in substantial errors in values of $\tilde{k}_{\text{XH}_2}$; for this reason it may be useful to note that the ratio of the values of k at the pH optima, $k_{\text{MPDS}}/k_{\text{2PDS}} = 14$; for the corresponding reactions of actinidin, which do not exhibit co-operativity, the ratio of the values of k at the pH optima $k_{\text{MPDS}}/k_{\text{2PDS}} = 1.3$, a value closely similar to the value of $\tilde{k}_{\text{MPDS}}/\tilde{k}_{\text{2PDS}}$ (1.2).

Fig. 8. pH-dependence of the u.v.-absorption of methyl 2-pyridyl disulphide in aqueous media at 25 °C and $I = 0.1$

(a) pH-dependent u.v. spectra traced from the recorder chart (0–1A range) of a Cary 118C spectrophotometer. pH values: (1) 1.42; (2) 1.89; (3) 2.46; (4) 2.91; (5) 3.55; (6) 3.95; (7) 7.33. (b) pH-dependence of ϵ_{307} for which the fitted line is theoretical for $\text{p}K_{\text{a}} = 2.8$.

determining the way in which binding effects and the effects of remote protonic dissociation, discussed above in relation to the kinetic studies on substrate hydrolysis, may be relayed differently to the catalytic sites of the two enzymes.

Concluding comments

Considerable differences in catalytic behaviour and catalytic-site chemistry exist between actinidin and papain despite their virtually superimposable crystal

structures in and around the catalytic-site region. Some differences in behaviour may be due to remote effects but others may be due to differences in structure near the catalytic site not detected readily by classical crystallography. These differences appear to be concerned with protein dynamics through which the catalytic site geometry may be controlled, and it remains to be seen whether protein motions of the type detected by chemical reactivity can be characterized structurally by currently developing techniques such as X-ray diffuse scattering analysis (Artymiuk, 1987; Doucet & Benoit, 1987). The problems encountered in attempting to correlate the structure and properties of actinidin and papain suggest that certain functional consequences of structural changes in enzymes produced by site-directed mutagenesis may not easily be predicted even when crystal structures of the native and mutant enzymes are available. Relatively subtle motions and field effects in the protein may be sufficiently perturbed by structural differences in mutant enzymes to produce substantial differences in catalytic-site chemistry, even though the structural basis for the differences may not be readily apparent from crystal structures. If mutant enzymes are assessed only by limited conventional steady-state analysis of the catalysed reaction together with comparison of the crystal structures of native and mutant enzymes, much of the potential value of site-directed mutagenesis in the elucidation of mechanism may not be realized. It is clearly vital to subject at least key mutant enzymes to detailed mechanistic investigation, using a wide range of techniques. Study of the chemical reactivity characteristics of catalytic-site residues is probably one of the most sensitive and most incisive methods for assessing changes of potential relevance for catalytic mechanism for which a structural basis must then be sought.

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