Identification of signalling and non-signalling binding contributions to enzyme reactivity

Alternative combinations of binding interactions provide for change in transition-state geometry in reactions of papain

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1. 2-(*N'*-Acetyl-L-phenylalanyl)hydroxyethyl 2'-pyridyl disulphide (compound V) was synthesized, and a study of the pH-dependence of the second-order rate constant (*k*) for its reaction with the catalytic-site thiol group of papain (EC 3.4.22.2) was used to evaluate the consequences for transition-state geometry of the presence of a hydrophobic occupant for the *S*₂ subsite of the enzyme in the absence of the N-H component of the *P*₁-*P*₂ amide bond. 2. Comparison of the pH-dependences of *k* for reactions of compound (V), 2-(acetamido)ethyl 2'-pyridyl disulphide (compound I) and 2-(acetoxy)ethyl 2'-pyridyl disulphide (compound III) with the cysteine-proteinase minimal catalytic-site model, benzimidazol-2-ylmethanethiol, established the activation of all of these pyridyl disulphides by hydronation and that their reactivities are relatively insensitive to structural change in the non-pyridyl part of the molecule. The marked differences in their reactivities towards papain therefore derive from binding, either directly, or indirectly via signalling mechanisms. 3. Comparison of the kinetic data for the reaction of papain with compound (V) with those for analogous reactions with reactivity probes that provide opportunities for a variety of binding interactions in the *S*₁-*S*₂ intersubsite region and in the *S*₂ subsite itself lead to the following conclusions: (a) the (Gly-66) N-H...O=C< (*P*₁-*P*₂ ester) interaction of papain with compound (III) provides for better binding relative to that for a probe with a simple hydrocarbon side chain, but no signalling to the catalytic site to provide a (His-159)-ImH⁺-assisted transition state; (b) when this interaction is augmented either by a (*P*₁-*P*₂ amide) N-H...O=C< (Asp-158) interaction (compound I) or hydrophobic *P*₂/*S*₂ contacts (compound V), signalling to the catalytic region occurs to provide the assisted transition state; (c) when both the *P*₂/*S*₂ contacts and the interaction involving Gly-66 exist, provision additionally of the (*P*₁-*P*₂ amide) N-H...O=C< (Asp-158) interaction [as in 2-(*N'*-acetyl-L-phenylalanyl)amino)ethyl 2'-pyridyl disulphide] serves only to assist the binding without an additional signalling effect. 4. Such studies promise to allow binding interactions that merely locate substrates in appropriate enzyme loci to be distinguished from those that transmit signals with a chemical consequence to catalytic sites.

INTRODUCTION

One of the least well-understood aspects of enzyme catalysis is the coupling of binding interactions involving non-scissile parts of substrates with catalytic-site chemistry. For many enzyme reactions it is conceivable, perhaps probable, that a proper description of the catalytic mechanism will lie between what Knowles (1987) refers to as the two linguistic extremes whereby recognition and catalysis are described either completely separately or, as in the classical treatments by Polanyi (1921), Haldane (1930) and Pauling (1946), as a single phenomenon.

The establishment of general concepts of binding-site-catalytic-site coupling requires studies on a variety of enzyme-substrate types. At one end of the mechanistic spectrum lie enzymes such as tyrosyl-tRNA synthetase that catalyse nucleophilic attack of one substrate on another by means of an array of differential binding interactions but without the intervention of catalytic-site

acids, bases or nucleophiles (see Leatherbarrow *et al.*, 1985; Wells & Fersht, 1986; Fersht *et al.*, 1986). Potentially more complex mechanistic situations arise where, in addition to the existence of substantial opportunities for differential binding interactions involving non-scissile parts of the substrate, a series of chemical events involving both enzyme and substrate occur. Many proteinases might be expected to come into this category, and the concept of functional coupling of binding and catalytic sites, despite their spatial separation in such enzymes, has received support both from steady-state kinetic evaluation of trypsin mutants (Craik *et al.*, 1985) and subtilisin mutants (Estell *et al.*, 1986; Wells *et al.*, 1987) and from stopped-flow kinetic studies on papain (EC 3.4.22.2), the most thoroughly characterized of the cysteine proteinases (for reviews see Baker & Drenth, 1987; Brocklehurst, 1987; Brocklehurst *et al.*, 1987b). The studies on papain have demonstrated a change in transition-state geometry promoted by binding interactions (Brocklehurst *et al.*, 1988a,b). Thus: (i) when

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(m.p. 128–131 °C) was synthesized by oxidation of 2-mercaptoethanol with H_2O_2 to provide 2-hydroxyethyl 2'-hydroxyethanethiolsulphonate followed by reaction with pyridine-2-thione as described by Brocklehurst *et al.* (1988b). 2-(*N'*-Acetyl-L-phenylalanyl)hydroxyethyl 2'-pyridyl disulphide (compound V) was synthesized by the route shown in Scheme 1. 2-Hydroxyethyl 2'-pyridyl disulphide hydrochloride (0.67 g, 3 mmol) was converted into the free base (a yellow oil) by treatment with NaHCO_3 (0.42 g, 5 mmol). This was extracted immediately into chloroform (3 × 20 ml), washed with water and dried over anhydrous MgSO_4 . After removal of solid material by filtration, the solvent was removed by rotary evaporation *in vacuo*. The oily residue was dissolved in methylene dichloride (10 ml) and was treated with *N*-t-butyloxycarbonyl-L-phenylalanine *N'*-hydroxy-succinimide ester (0.79 g, 3 mmol) and 4-(*NN*-dimethyl-amino)pyridine (0.08 g, 1 mmol). The reaction mixture was left to stand for 48 h at room temperature (approx. 22 °C) and was then washed in a separating funnel, first with saturated aq. NaHCO_3 and then with water. The methylene dichloride layer was dried over anhydrous MgSO_4 . After removal of the drying agent by filtration, the solvent was removed by rotary evaporation *in vacuo*. The residue was dissolved in methylene dichloride (10 ml) at 15 °C, and trifluoroacetic acid (10 ml) was added. The reaction mixture was left to stand at this temperature for 1 h and then evaporated to dryness *in vacuo*, the temperature being kept below 20 °C. The residue was partitioned between aq. NaHCO_3 and ethyl acetate and the ethyl acetate layer was dried over anhydrous MgSO_4 . After removal of the drying agent by filtration, the filtrate was treated sequentially with triethylamine (0.84 ml, 6 mmol) and acetic anhydride (0.5 ml, 5 mmol), and the mixture was stirred for 15 min at room temperature. Rotary evaporation *in vacuo* left an oil, which was purified by column chromatography. Elution from a column (20 cm × 1.5 cm) of silica gel (Kieselgel 60, 70–230 mesh; Merck) with chloroform as eluent provided a colourless oil as a chromatographically

and N–H 3275 cm^{-1} ; n.m.r. data, δ (p.p.m.) ($[\text{H}^2\text{H}]$ chloroform) 8.4–7.0 (9H, m, aromatic rings), 5.9 (d, NH, exchange $^2\text{H}_2\text{O}$), 4.8 (1H, q, HN–CH–CH₂–), 4.3 (2H, t, –CH₂–), 3.1 (2H, d, –CH₂Ph), 2.9 (2H, t, –CH₂–) and 1.95 (3H, s, CH₃CO–).

Kinetics

All reactions were studied at 25 °C and 10.1 in solutions containing 1 mM-EDTA with Dionex stopped-flow equipment connected to a Tektronix 5103N oscilloscope and interfaced with a Commodore 4032 microcomputer. Some kinetic runs in each set were recorded by photographing the oscilloscope screen, and conventional first-order analysis of these records always provided values of rate constants in good agreement with those produced by the on-line microcomputer. Release of pyridine-2-thione consequent on reaction of the enzyme thiol group with the 2-pyridyl disulphide probe reagents was monitored at 343 nm and quantified by using $\epsilon_{343} = 8.0 \times 10^3 / (1 + K/[\text{H}^+]) \text{ M}^{-1} \cdot \text{cm}^{-1}$, where $\text{p}K = 9.8$ (Brocklehurst & Little, 1973; Stuchbury *et al.* 1975). The buffers were as described by Brocklehurst *et al.* (1987a). Because of the high reactivity of papain towards compound (V) it was necessary at most pH values to carry out the kinetic analysis under equimolar (2–3 μM) second-order conditions even when using the stopped-flow technique. At both extremes of the pH range used, however, it was possible to carry out reactions also under pseudo-first-order conditions with [papain] = 2–3 μM and [compound (V)] = 30–60 μM . In these pH regions similar values of the second-order rate constant (k) were obtained by both pseudo-first-order and second-order kinetic methods (see Fig. 1). The pH-dependence of k and of the individual contributions from the various hydronic states [the International Union of Pure and Applied Chemistry (1986) suggests that the ion H^+ be called the hydron when an individual isotope is not being specified] was evaluated by using the eight-parameter (four $\text{p}K_a$) rate equation (eqn. 1) discussed by Brocklehurst & Brocklehurst (1988) (see also the Results and discussion section):

$$k = \frac{\tilde{k}_{\text{XH}_3}}{1 + \frac{[\text{H}^+]}{K_{\text{XH}_4}} + \frac{K_{\text{XH}_3}}{[\text{H}^+]} + \frac{K_{\text{XH}_3}K_{\text{XH}_2}}{[\text{H}^+]^2} + \frac{K_{\text{XH}_3}K_{\text{XH}_2}K_{\text{XH}}}{[\text{H}^+]^3}} + \frac{\tilde{k}_{\text{XH}_2}}{1 + \frac{[\text{H}^+]^2}{K_{\text{XH}_4}K_{\text{XH}_3}} + \frac{[\text{H}^+]}{K_{\text{XH}_3}} + \frac{K_{\text{XH}_2}}{[\text{H}^+]} + \frac{K_{\text{XH}_2}K_{\text{XH}}}{[\text{H}^+]^2}} + \frac{\tilde{k}_{\text{XH}}}{1 + \frac{[\text{H}^+]^3}{K_{\text{XH}_4}K_{\text{XH}_3}K_{\text{XH}_2}} + \frac{[\text{H}^+]^2}{K_{\text{XH}_3}K_{\text{XH}_2}} + \frac{[\text{H}^+]}{K_{\text{XH}_2}} + \frac{K_{\text{XH}}}{[\text{H}^+]}} + \frac{\tilde{k}_{\text{X}}}{1 + \frac{[\text{H}^+]^4}{K_{\text{XH}_4}K_{\text{XH}_3}K_{\text{XH}_2}K_{\text{XH}}} + \frac{[\text{H}^+]^3}{K_{\text{XH}_3}K_{\text{XH}_2}K_{\text{XH}}} + \frac{[\text{H}^+]^2}{K_{\text{XH}_2}K_{\text{XH}}} + \frac{[\text{H}^+]}{K_{\text{XH}}}} \quad (1)$$

homogeneous product, which crystallized on storage for 2 days at 4 °C. It was recrystallized from cold ethyl acetate to which hexane was added to turbidity, and then had m.p. 56 °C and $[\alpha]_{\text{D}}^{20} + 5.9 \pm 0.1^\circ$ (*c* 0.054 in methanol). This value of the specific rotation is similar to that (+7.0°) which characterizes compound (IV) (Brocklehurst *et al.*, 1987a). The mass spectrum gave m/z 376 [M^+]; $\text{C}_{16}\text{H}_{22}\text{N}_2\text{O}_5\text{S}_2$ requires M^+ 376. A sample produced the predicted yield of pyridine-2-thione consequent upon thiolysis with 2-mercaptoethanol at pH 7.0, deduced by spectral analysis at 343 nm (ϵ_{343} 8080 $\text{M}^{-1} \cdot \text{cm}^{-1}$; Stuchbury *et al.*, 1975). The thiolysis analysis, the mass spectrum and the following spectroscopic data established the identity of the product as compound (V): i.r. data, C=O 1650 cm^{-1} and 1740 cm^{-1} ,

and a BBC Microcomputer (model B) fitted with a 6502 second processor. Kinetic analysis of the reactions of benzimidazol-2-ylmethanethiol with various disulphides was carried out at selected pH values under pseudo-first-order conditions with [thiol] = 3–5 μM and [disulphide] = 50–300 μM .

RESULTS AND DISCUSSION

Reactivity-probe design, synthesis and characterization

2-(*N'*-Acetyl-L-phenylalanyl)hydroxyethyl 2'-pyridyl disulphide (compound V) was designed as a chromogenic thiol-specific two-hydronic-state reactivity probe (Brocklehurst *et al.*, 1987a) containing a potential occupant for the S_2 subsite of papain with the capacity for acceptance of a hydrogen bond from the N–H of Gly-

Scheme 2. Schematic drawing showing the bonding of the P_1 - P_2 carbonyl oxygen atom of compound (V) to the N-H of Gly-66 of papain and of the benzyl side chain in the S_2 subsite

The existence of these two interactions appears to provide for the transition state depicted involving both partners of the $-S^-/ImH^+$ ion-pair and the non-hydrated form of compound (V).

66 but without the capacity to act as a hydrogen-bond donor to the backbone carbonyl oxygen atom of Asp-158 (see Scheme 2). Its synthesis (Scheme 1) involved acylation of 2-hydroxyethyl 2'-pyridyl disulphide with *N*-*t*-butyloxycarbonyl-L-phenylalanine *N'*-hydroxy-succinimide ester, followed by the removal of the *t*-butyloxy group in trifluoroacetic acid and acetylation of the freed amino group with acetic anhydride. The similarity in the values of $[\alpha]_D^{20}$ for compounds (IV) and (V) together with the observation of single-phase progress curves for complete reaction with papain under equimolar conditions suggest that racemization did not occur to an appreciable extent during the acylation of 2-hydroxyethyl 2'-pyridyl disulphide, despite the fact that the reaction was allowed to proceed for 2 days at room temperature, conditions much harsher than those used to acylate the amino analogue in the synthetic route to compound (IV) (2 h at 0 °C; Brocklehurst *et al.*, 1987a).

The pK_a of the conjugate acid of compound (V) in which the pyridyl ring is hydronated was determined to be 2.4 (at 25 °C and 1.0.1) by the pH-dependent change in A_{308} . The electronic spectra of disulphides containing the 2-mercaptopyridine group undergo large pH-dependent changes in u.v. absorption around 300 nm (see Salih *et al.*, 1987). Study of the pH-dependence of k for the reactions of compounds (I), (III) and (V) with benzimidazol-2-ylmethanethiol (Table 1) established that (a) all of these disulphides are 'two-hydronic-state electrophiles' (see Brocklehurst *et al.*, 1987a) in that their reactions with a mercaptan with nucleophilic character in two hydronic states are characterized by three reactive hydronic states with a rate minimum at pH 6–7 as in the pH- k profile reported for the analogous reaction of compound (I) (Brocklehurst *et al.*, 1988a), and (b) the intrinsic electrophilic reactivities of these compounds, as would be expected, are relatively insensitive to structural change in the non-pyridyl part of the disulphide. Thus the considerable differences in

Table 1. Demonstration of a rate minimum at pH approx. 6 and of the relative insensitivity of reactivity to side-chain structure in the reactions of three 2-pyridyl disulphides with the minimal cysteine-proteinase catalytic-site model benzimidazol-2-ylmethanethiol at 25 °C and 1.0.1

A complete pH- k profile for the reaction with compound (I) was reported by Brocklehurst *et al.* (1988a).

pH	$10^{-3} \times k \text{ (M}^{-1} \cdot \text{s}^{-1}\text{)}$		
	Compound (I)	Compound (III)	Compound (V)
3.30	1.1	1.3	1.3
4.40	1.1	1.3	1.3
5.03	0.9	1.2	1.1
6.23	0.3	0.5	0.5
7.06	0.5	0.65	0.7
9.90	7.9	8.2	9.9

reactivity displayed by compounds (I)–(V) in their reactions with papain (see below) must derive from binding interactions and their consequences for catalytic-site chemistry.

pH-dependent kinetics of the reaction of papain with 2-(*N'*-acetyl-L-phenylalanyl)hydroxyethyl 2'-pyridyl disulphide (compound V)

The main features of the shape of the pH- k profile for this reaction (Fig. 1) are similar to those of the analogous profile for the reaction of papain with compound (IV), which contains an intact P_1 - P_2 amide bond as well as the L-phenylalanine side chain at P_2 (Brocklehurst *et al.*, 1988b). Thus in both profiles there is a striking rate maximum at pH 6–7 and a plateau of much lower reactivity approached at pH > 10. In these two pH regions the values of k for the reaction with compound (V) are about 5 times less than the values for the reaction with compound (IV). The shape of the profile in Fig. 1 contrasts markedly with the shapes of the corresponding profiles for the reactions of papain with featureless probes containing only a simple hydrocarbon side chain [e.g. compound (II)], and with compound (III), the probe that lacks an L-phenylalanine residue at P_2 and contains a P_1 - P_2 ester bond instead of a P_1 - P_2 amide bond (Fig. 2, curves B and C). In these cases there is a rate minimum at pH 6–7 instead of a rate maximum. The shape of the profile for the reaction with compound (I), which contains a P_1 - P_2 amide bond but which lacks an L-phenylalanine residue at P_2 (Fig. 2, curve A), is intermediate between the shape of that in Fig. 1 and those in Fig. 2 (curves B and C) in that there is rate maximum at pH 6, but this is

Fig. 1. pH-dependence of the second-order rate constant (k) for the reaction of papain with 2-(N' -acetyl-L-phenylalanyl)-hydroxyethyl 2'-pyridyl disulphide (compound V) at 25 °C and I 0.1 in aqueous buffers

The points are experimental (□, determined under equimolar second-order conditions; ■, determined under pseudo-first-order conditions with the probe reagent in excess), and the continuous line is theoretical for eqn. (1) with the following values of the characterizing parameters ($\tilde{k}$ in $\text{M}^{-1}\cdot\text{s}^{-1}$): $\tilde{k}_{\text{XH}_3} = 9.0 \times 10^5$, $\tilde{k}_{\text{XH}_2} = 1.2 \times 10^6$, $\tilde{k}_{\text{XH}} = 6.0 \times 10^5$, $\tilde{k}_X = 1.15 \times 10^5$, $\text{p}K_{\text{XH}_4} = 3.9$, $\text{p}K_{\text{XH}_3} = 4.2$, $\text{p}K_{\text{XH}_2} = 8.2$, and $\text{p}K_{\text{XH}} = 9.5$; the broken lines A–D correspond to the contributions to k of the individual hydronic states of the reaction that are provided by the terms in eqn. (1) associated with $\tilde{k}_{\text{XH}_3}$ – $\tilde{k}_X$ respectively.

less well developed relative to the reactivity at high pH. The characterizing parameters (macroscopic $\text{p}K_a$ values and pH-independent rate constants, $\tilde{k}$) for the various hydronic states (designated X, XH, XH_2 etc. to indicate their relative stoichiometries in hydrons) of the reactions of papain with probe (V) are compared with those for the reactions with probes (I)–(IV) (Brocklehurst *et al.*, 1988b) in Tables 2 and 3.

The $\text{p}K_a$ values of Fig. 1

The steepness of the acid limb of the profile requires two $\text{p}K_a$ values close to 4 to provide a fit to the data. This is a phenomenon that is characteristic of many reactions of papain and of $k_{\text{cat.}}/K_m$ for catalysis. The involvement of two ionizations each with $\text{p}K_a$ approx. 4 in the behaviour of papain was noted for both reactivity (Brocklehurst & Little, 1972) and catalytic activity (Sluyterman & Wijdenes, 1973) and has been the subject of many subsequent studies (e.g. Shipton *et al.* 1975; Lewis *et al.*, 1978; Brocklehurst & Malthouse, 1980; Salih *et al.*, 1987). The alkaline limb is fitted in Fig. 1 by two $\text{p}K_a$ values of 8.2 and 9.5 respectively. A good fit to the data is obtained also when a single $\text{p}K_a$ of 9.0 is used instead. Evidence is beginning to accumulate, however (see Brocklehurst *et al.*, 1988b), that catalytic-site chemistry and catalytic activity of papain may be influenced by two hydronic dissociations with $\text{p}K_a$ valued > 7 . One of these has $\text{p}K_a$ approx. 8.2 and the other $\text{p}K_a$ approx. 9.5. In some cases one or other is dominant and is clearly discerned. For example, in curve A in Fig. 2 k clearly decreases in alkaline media mainly across a $\text{p}K_a$ of approx. 8 and certainly not across a

Fig. 2. pH-dependence of k for the reaction of papain with (curve A) 2-(acetamido)ethyl 2'-pyridyl disulphide (compound I), (curve B) 2-(acetoxy)ethyl 2'-pyridyl disulphide (compound III) and (curve C) n-propyl 2-pyridyl disulphide (compound II)

The conditions are as described for Fig. 1. The arrows define the pH ranges over which data points were collected (see Brocklehurst *et al.*, 1988b). The continuous lines are theoretical for eqn. (1) in the case of curves A and B, and in the case of curve C for the amended form of eqn. (1) appropriate to a three-reactive-hydronic-state model, with the following values of the characterizing parameters (with $\tilde{k}$ in $\text{M}^{-1}\cdot\text{s}^{-1}$): curve A, $\tilde{k}_{\text{XH}_3} = 1.0 \times 10^3$, $\tilde{k}_{\text{XH}_2} = 2.95 \times 10^4$, $\tilde{k}_{\text{XH}} = 2.95 \times 10^4$, $\tilde{k}_X = 1.95 \times 10^4$, $\text{p}K_{\text{XH}_4} = 2.48$ (the value of the $\text{p}K_a$ of $\text{CH}_3\text{-CO-NH-CH}_2\text{-CH}_2\text{-S-S-2-PyH}^+$), $\text{p}K_{\text{XH}_3} = 3.7$, $\text{p}K_{\text{XH}_2} = 3.7$, and $\text{p}K_{\text{XH}} = 8.1$; curve B, $\tilde{k}_{\text{XH}_3} = 3.5 \times 10^4$, $\tilde{k}_{\text{XH}_2} = 1.0 \times 10^4$, $\tilde{k}_{\text{XH}} = 5.75 \times 10^3$, $\tilde{k}_X = 1.65 \times 10^4$, $\text{p}K_{\text{XH}_4} = \text{p}K_{\text{XH}_3} = 3.75$, $\text{p}K_{\text{XH}_2} = 5.0$, and $\text{p}K_{\text{XH}} = 9.5$; curve C, $\tilde{k}_{\text{XH}_2} = 4.4 \times 10^4$, $\tilde{k}_{\text{XH}} = 8.0 \times 10^2$, $\tilde{k}_X = 1.8 \times 10^3$, $\text{p}K_{\text{XH}_3} = \text{p}K_{\text{XH}_2} = 4.0$, and $\text{p}K_{\text{XH}} = 9.5$.

$\text{p}K_a$ of 9.5, whereas for the increases in k in curves B and C in Fig. 2 the opposite is true, i.e. the dominant $\text{p}K_a$ is clearly approx. 9.5.

One of these $\text{p}K_a$ values probably characterizes the hydronic dissociation of the catalytic-site ion-pair state $\text{S}^-/\text{-ImH}^+ \rightleftharpoons \text{S}^-/\text{-Im}$ and the other must be assigned to another site in the enzyme (possibly His-81) that controls catalytic-site geometry, possibly mediated through binding interactions (Brocklehurst *et al.*, 1988b). Given the existence of these two kinetically influential $\text{p}K_a$ values, the possibility exists that some reactions with appropriate ratios of reactivities of the various reactive hydronic states will appear to be controlled by a single ionization with an apparent $\text{p}K_a$ value between approx. 8.2 and approx. 9.5. The variability of observed $\text{p}K_a$ values characteristic of what is sometimes apparently a single ionization of the free enzyme molecule with change in probe or substrate structure resulting from overlapping ionizations has been explored previously (Brocklehurst *et al.*, 1983).

The $\tilde{k}_{\text{XH}}/\tilde{k}_X$ ratio as a criterion for assessment of binding-site-catalytic-site signalling

The second-order rate constants ($\tilde{k}$) characteristic of individual hydronic states, $\tilde{k}_{\text{XH}_n}$ ($n > 1$) and $\tilde{k}_X$, the reference state at high pH, are usefully considered as ratios of k_{+2} and K_r [eqns. (2)–(5); eqn. (5) probably

Table 2. Effects of structural variation in substrate-derived 2-pyridyl disulphide reactivity probes (R-S-S-2-Py) on the characteristics of their reactions with the catalytic-site thiol group of papain at 25 °C and / 0.1

The various hydronic states are designated X-XH₄ to indicate their relative stoichiometries in hydrons; the XH₄ state is unreactive, as is the XH₃ state for the reaction with probe (II); the shapes of the pH-*k* profiles are shown in Fig. 2 for reactions with probes (I)-(III) and in Fig. 1 for the reaction with probe (V). The profile for the reaction with probe (IV) has a shape similar to that of Fig. 1 (see Brocklehurst *et al.*, 1988b).

Probe	Macroscopic p <i>K</i> _a values				pH-independent rate constants (M ⁻¹ ·s ⁻¹)			
	p <i>K</i> _{XH1}	p <i>K</i> _{XH3}	p <i>K</i> _{XH2}	p <i>K</i> _{XH}	10 ⁻⁴ × <i>k̃</i> _{XH3}	10 ⁻⁴ × <i>k̃</i> _{XH2}	10 ⁻⁴ × <i>k̃</i> _{XH}	10 ⁻⁴ × <i>k̃</i> _X
(IV) <div>$\text{CH}_3\text{-}\overset{\text{O}}{\parallel}\text{C-NH-CH}(\text{CH}_2\text{Ph})\text{-}\overset{\text{O}}{\parallel}\text{C-NH-}[\text{CH}_2]_2\text{-S-S-2-Py}$</div>	3.9	4.1	8.25	9.5	300	610	230	60
(V) <div>$\text{CH}_3\text{-}\overset{\text{O}}{\parallel}\text{C-NH-CH}(\text{CH}_2\text{Ph})\text{-}\overset{\text{O}}{\parallel}\text{C-O-}[\text{CH}_2]_2\text{-S-S-2-Py}$</div>	3.9	4.2	8.2	9.5	90	120	60	11.5
(I) <div>$\text{CH}_3\text{-}\overset{\text{O}}{\parallel}\text{C-NH-}[\text{CH}_2]_2\text{-S-S-2-Py}$</div>	2.48*	3.7	3.7	8.1†	0.1	2.95	2.95	1.95
(III) <div>$\text{CH}_3\text{-}\overset{\text{O}}{\parallel}\text{C-O-}[\text{CH}_2]_2\text{-S-S-2-Py}$</div>	3.75	3.75	5.0*	9.5	3.5	1.0	0.58	1.65
(II) <div>$\text{CH}_3\text{-}[\text{CH}_2]_2\text{-S-S-2-Py}$</div>	–	4.0	4.0	9.5	–	4.4	0.08	0.18

* p*K*_a 2.48 characterizes hydronic dissociation from the conjugate acid of probe (I) from the pyridyl nitrogen atom; p*K*_a 5.0 characterizes an additional sigmoid wave in this profile.

† The common features of most of these profiles are two co-operative hydronic dissociations with p*K*_a values of approx. 4 and two in alkaline media with p*K*_a values of approx. 8.2 and 9.5, but for the reaction with probe (I) the p*K*_a of 9.5 cannot be discerned, possibly because of the small amplitude of the sigmoid wave linking the XH and X states.

Table 3. Effects of various combinations of the following interactions on the relative reactivities of individual hydronic states ($X-XH_2$) of the reactions of papain with substrate-derived 2-pyridyl disulphides R-S-S-2-Py: (a) interaction of the L-phenylalanine side chain in the S_2 subsite, (b) hydrogen-bonding of the backbone N-H of Gly-66 to the P_1-P_2 subsite carbonyl oxygen atom and (c) hydrogen-bonding of the P_1-P_2 amide N-H to the backbone carbonyl oxygen atom of Asp-158

Values given in parentheses reflect other phenomena not discussed in the present paper that derive from additional hydronic dissociations (see Table 2); [1] denotes unity of definition.

Probe	Side chain (R)	Postulated binding interaction(s)	$\tilde{k}_{XH}$		$\tilde{k}_{XH_2}$		$\tilde{k}_{X(\text{probe})}$		$\tilde{k}_{XH(\text{probe})}$		$\tilde{k}_{XH_2(\text{probe})}$		$\tilde{k}_{XH(\text{probeII})}$		$\tilde{k}_{XH_2(\text{probeII})}$	
			$\tilde{k}_X$	$\tilde{k}_{XH}$	$\tilde{k}_X$	$\tilde{k}_{XH_2}$	$\tilde{k}_{X(\text{probeII})}$	$\tilde{k}_{X(\text{probe})}$	$\tilde{k}_{XH(\text{probeII})}$	$\tilde{k}_{XH(\text{probe})}$	$\tilde{k}_{XH_2(\text{probeII})}$	$\tilde{k}_{XH_2(\text{probe})}$	$\tilde{k}_{XH(\text{probeII})}$	$\tilde{k}_{XH(\text{probe})}$	$\tilde{k}_{XH_2(\text{probeII})}$	$\tilde{k}_{XH_2(\text{probe})}$
(IV)	$\text{CH}_3-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{CH}(\text{CH}_2\text{Ph})-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-[\text{CH}_2]_2-$	(a), (b) and (c)	3.8	10.2*	36	333	397	1052†	2875	7625‡						
(V)	$\text{CH}_3-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{CH}(\text{CH}_2\text{Ph})-\overset{\text{O}}{\parallel}\text{C}-\text{O}-[\text{CH}_2]_2-$	(a) and (b)	5.2	10.4*	7	64	103	207†	750	1500‡						
(I)	$\text{CH}_3-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-[\text{CH}_2]_2-$	(b) and (c)	1.5*	(1.5)	1.2	11	5†	(5)	37‡	(37)						
(III)	$\text{CH}_3-\overset{\text{O}}{\parallel}\text{C}-\text{O}-[\text{CH}_2]_2-$	(b)	0.35*	(0.6)	[1]	9	[1]†	[1]	7‡	(13)						
(II)	$\text{CH}_3-[\text{CH}_2]_2-$	None	0.4*	(24)	0.1	[1]	0.1†	(8)	[1]‡	[1]						

* These five values provide a comparison of the relative reactivities in the pH region 6–7, where k is a maximum [$\tilde{k}_{XH_2}$ for probes (IV) and (V) and $\tilde{k}_{XH}$ for probe (I)] or a minimum $\tilde{k}_{XH}$ for probes (II) and (III), and in the plateau region at high pH; the values of $\tilde{k}_{XH}$ for probes (IV) and (V) relate to the intermediate hydronic state on the alkaline limb of the pH- k profile bounded by pK_a values of approx. 8.2 and 9.5 (see Table 2).

† and ‡ Similarly, values within each of these two sets of five should be compared to relate the reactivities in the regions of the rate maxima and minima (pH 6–7) to change in side-chain structure.

applies when $k < 1 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Brocklehurst, 1979)] to permit binding effects as such (reflected in K_r) to be distinguished in concept from consequential changes in transition-state geometry in particular hydronic states (reflected in k_{+2}) (Brocklehurst *et al.*, 1987a, 1988b):

$$k = k_{+1}k_{+2}/(k_{-1} + k_{+2}) \quad (3)$$

$$k = k_{+2}/K_r \quad (4)$$

$$K_r = k_{-1}/k_{+1} \quad (5)$$

Thus for the reactions of cysteine proteinases with 2-pyridyl disulphide reactivity probes a change in probe structure that provides for a uniform change in k over the entire pH range (i.e. no change in the shape of the pH- k profile) is most simply interpreted as an effect on K_r . When the structural change affects differentially the reactivities of individual hydronic states, leading to a change in profile shape, however, a change in transition-state structure, reflected in k_{+2} , needs to be considered. In particular, a structural change leading to a substantial increase in $\tilde{k}_{\text{XH}_n}/\tilde{k}_\text{X}$ (often when $n = 1$ or 2), which may in some cases change $\tilde{k}_{\text{XH}_n}/\tilde{k}_\text{X}$ from a value less than 1 (producing a rate minimum at pH approx. 6–7) to a value greater than 1 (producing a rate maximum in this pH region) is strongly suggestive of a change in transition-state geometry permitted or promoted by particular binding interactions involving the non-scissile parts of the substrate-derived probe molecule.

The principal feature of a cysteine-proteinase catalytic site is an interactive system comprising the side chains of a cysteine residue and a histidine residue of which an intimate thiolate-imidazolium ion pair is probably a substantial component (see Brocklehurst *et al.*, 1987b). In the most proton-deficient, X, state (at high pH) containing S^- and Im , the transition state (VI) for reaction with a 2-pyridyl disulphide involves some charge development on the pyridyl nitrogen atom leading to the expulsion of the 2-mercaptopyridine anion ($\text{p}K_\text{a}$ 9.8; Brocklehurst & Little, 1973). Transition-state stabilization may be achieved either when the pyridyl nitrogen atom is hydronated in the reactant state leading to the neutral pyridinethione as the immediate product via transition state (VII), or by donation of a hydrogen bond from a neighbouring acidic chain, most reasonably the catalytic-site ImH^+ cation, through transition state (VIII), with post-transition-state hydron transfer [see Brocklehurst *et al.* (1988a) for a discussion of the inverse solvent kinetic isotope effect, which probably rules out hydron transfer in the transition state]. In simple cases where additional hydronations (not shown) do not affect chemical reactivity, transition state (VIII) represents an XH state in which the imidazolium ion and the pyridyl nitrogen atom make a hydrogen-bonding-cum-electrostatic contact, and transition state (VII)

represents an XH_2 state in which the thiolate anion component of the ion-pair attacks the pyridyl disulphide cation. When binding interactions do not provide for the contact shown in transition state (VIII), $\tilde{k}_\text{X} > \tilde{k}_{\text{XH}}$ and the pH- k profile contains a minimum at pH 6–7 because of the high reactivity associated with transition states like (VII). This is the situation for 'featureless' probes, e.g. where $\text{R} = \text{n-propyl}$ (II), and for probe (III), where $\text{R} = \text{CH}_3\text{-CO-O-}$ (see Tables 2 and 3 and Brocklehurst *et al.*, 1988b). When binding interactions do permit effective reaction through transition state (VIII) (see below), the result is a striking maximum in the pH- k profile at pH 6–7, like that in Fig. 1 (see also Tables 2 and 3 and Brocklehurst *et al.*, 1988a).

Since reactions involving some probes [e.g. (II) and (III)] do not exhibit a maximum with $\tilde{k}_{\text{XH}} > \tilde{k}_\text{X}$, there must be some change in the conformation of the transition state in those reactions with probes that do [e.g. (I), (IV) and (V)]. The ratio $\tilde{k}_{\text{XH}}/\tilde{k}_\text{X}$ (or in some cases $\tilde{k}_{\text{XH}_2}/\tilde{k}_\text{X}$; see below) provides an index of the effectiveness with which the stabilization of the transition state promoted by particular binding interactions is effected. It is not possible at present to ascertain whether these binding effects constitute virtually an 'all-or-none' phenomenon in which either the $\text{ImH}^+ \cdots \text{Py}$ hydrogen bond is made or it is not, or whether the quality of the hydrogen-bonding is affected by the binding, through change in bond angles and distances. In the former case an increase in the $\tilde{k}_{\text{XH}}/\tilde{k}_\text{X}$ or $\tilde{k}_{\text{XH}_2}/\tilde{k}_\text{X}$ ratio would reflect only an increased population of hydrogen-bonded transition states, and in the latter a quantitative interpretation would be more complex. It is necessary to compare appropriate sets of $\tilde{k}_{\text{XH}_2}/\tilde{k}_\text{X}$ and $\tilde{k}_{\text{XH}}/\tilde{k}_\text{X}$ ratios in reactions of papain because the reactivity of the ion-pair is modulated in some instances by another hydronic dissociation (see Tables 2 and 3). In these cases [e.g. for probes (IV) and (V)] reaction through the most effective and/or most populated hydrogen-bonded transition state is compared with the reaction in the X state by consideration of the $\tilde{k}_{\text{XH}_2}/\tilde{k}_\text{X}$ ratio. In other cases [e.g. for probes (I)–(III)] the $\tilde{k}_{\text{XH}}/\tilde{k}_\text{X}$ ratio is the appropriate index.

The binding and signalling components of papain-ligand systems

The simplest interpretation of the marked changes in $\tilde{k}_\text{X}$ (Table 2) [characteristic of reactions through transition state (VI)] with change in probe structure is that provision of various binding interactions increases the steady-state or quasi-equilibrium concentration of the enzyme-probe adsorptive complex by progressive decreases in K_r . Thus the ratio $\tilde{k}_{\text{X(probe)}}/\tilde{k}_{\text{X(probeII)}}$ (Table 3), which relates X-state reactivities to that of papain towards the probe with a simple hydrocarbon side chain, (a) is increased (from unity) approx. 10-fold by provision of the opportunity for the $(\text{Gly-66}) \text{N-H} \cdots \text{O}=\text{C} <$

(VI)

(VII)

(VIII)

(P_1 - P_2 amide) interaction (see Scheme 2), (b) is not increased further by provision also of the opportunity for the (P_1 - P_2 amide) $N-H \cdots O=C<$ (Asp-158) interaction, (c) is increased approx. 60-fold when both the Gly-66 interaction and good S_2/P_2 contacts exist, and (d) is increased approx. 300-fold when all three binding contacts would be predicted to occur. Thus binding appears to be better by about 5-fold when all three interactions can occur than when the possibility for the (P_1 - P_2 amide) $N-H \cdots O=C<$ (Asp-158) interaction is removed (probe V). When the S_2/P_2 interaction is removed, binding is decreased by 6-7-fold whether or not the (P_1 - P_2) $N-H \cdots O=C<$ (Asp-158) interaction is provided for (probes I and III) as long as the (Gly-66) $N-H \cdots O=C<$ (P_1 - P_2 amide/ester) interaction is maintained. When this interaction is removed (probe II), binding is decreased by 10-fold.

On the assumption that relative reactivities through transition state (VI) reflect mainly relative binding abilities of the probes (K_r effects), consideration of hydronic states capable of providing for reaction through transition state (VIII) should reveal binding-site-catalytic-site signalling effects (k_{+2} effects) resulting in $-ImH^+$ -assisted reaction as deviations from the analogous reactivity ratios in the X state. This is seen most clearly by considering reaction in the hydronic state for a particular probe reaction that provides for the rate maximum or minimum at pH 6-7, where the $-ImH^+$ -assisted reaction (transition state VIII) appears to be fully developed. For probes (I)-(III) this is the XH state and for probes (IV) and (V) it is the XH_2 state. Thus it is appropriate to consider $k_{XH_2(\text{probe})}/k_{XH(\text{probe II})}$ ratios for probes (IV) and (V) together with $k_{XH(\text{probe})}/k_{XH(\text{probe II})}$ ratios (Table 3) as a coherent set. As possibilities for binding are introduced, it is easy to distinguish those that produce comparable effects in both X and XH (or XH_2) states and those in which additional rate enhancements are produced in the appropriate XH or XH_2 state. Change of a simple hydrocarbon side chain (probe II) to one in which the (Gly-66) $N-H \cdots O=C<$ (P_1 - P_2 ester) interaction is possible (probe III) produces comparable increases in both X and XH state reactivities (i.e. no evidence for anything other than a K_r effect). When the possibility for the (P_1 - P_2) $N-H \cdots O=C<$ (Asp-158) interaction is added, however, but without S_2/P_2 contacts (probe I), there is a 5-fold increase in reactivity in the XH state but essentially no increase in the X state (Table 3), and this provides for the change in shape of the pH- k profile in which k_{XH}/k_X changes from a value less than 1 for probe (III) (a rate minimum at pH 6-7) to a value greater than 1 for probe (I) (a rate maximum at pH 6-7). Also, when the potential interaction involving Asp-158 is removed and a hydrophobic P_2/S_2 interaction is provided for probe (V), the rate enhancement over that provided by the structural features of probe (I) is 40-fold (i.e. 1500/37) in the XH_2 state ($k_{XH_2(\text{probe V})}/k_{XH(\text{probe II})} = 1500$) but only 6-fold (i.e. 64/11) in the X state ($k_{X(\text{probe V})}/k_{X(\text{probe II})} = 64$). Reinstatement of the interaction involving Asp-158 while retaining the P_2/S_2 interaction (probe IV) provides an additional 5-fold rate enhancement in both X and XH_2 states.

Concluding comments

The above analysis suggests that the (Gly-66) $N-H \cdots O=C<$ (P_1 - P_2 ester) interaction provides for

better binding but not for the signalling effect required to provide a transition state of type (VIII). When this interaction is augmented by either a (P_1 - P_2 amide) $N-H \cdots O=C<$ (Asp-158) interaction or hydrophobic P_2/S_2 contacts, signalling to the catalytic-site region occurs, providing reaction through transition state (VIII). When both the P_2/S_2 contacts and the interaction involving Gly-66 exist, provision additionally of the (P_1 - P_2 amide) $N-H \cdots O=C<$ (Asp-158) interaction serves only to assist the binding without an additional signalling effect.

It seems probable that various combinations of binding interactions will often prove crucial to efficient catalysis, particularly for enzymes with large binding sites that provide for a multiplicity of enzyme-substrate contacts. A recent example is provided by the steady-state kinetic study of the specificity of mesentericopeptidase (a proteinase closely related to subtilisin BPN') (Bratovanova & Petkov, 1987). Kinetic data for both hydrolysis and aminolysis of *N*-anthranilylated peptide *p*-nitroanilides suggest a stringent specificity for glycine at P'_1 and a preference for bulky hydrophobic residues at P'_2 , with synergism in the effects of binding in the S'_1 and S'_2 subsites. Glycine flanked on both sides in a substrate by bulky hydrophobic residues appears to be the minimum amino acid sequence for effective catalysis in subtilisin-like enzymes.

The results presented in the present paper demonstrate that, by the use of substrate-derived reactivity probes designed such that one part of the probe molecule can make selective binding contacts while the other can detect change in transition-state geometry, it is possible to distinguish binding effects and particular combinations of binding effects that provide signalling from those that serve merely to locate a ligand in the appropriate binding site of the enzyme.

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