Substrate-derived two-protonic-state electrophiles as sensitive kinetic specificity probes for cysteine proteinases

Activation of 2-pyridyl disulphides by hydrogen-bonding

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INTRODUCTION

The cysteine proteinases are proving to be a valuable enzyme family for assessing the effects of structural variation in the protein (see Kamphuis et al., 1985) on active-centre chemistry and catalytic mechanism (Brocklehurst, 1986, 1987; Willenbrock & Brocklehurst, 1984, 1985a,b, 1986). One of the most sensitive and direct ways of investigating catalytic-site chemistry and its modulation by ligand binding is the study of chemical reactivity characteristics. In particular, investigation of the reactivity of a catalytic-site nucleophile that plays a central role in the catalytic act is a prime target for investigation of the factors that help to determine transition-state geometries. In the cysteine proteinases the catalytic-site nucleophile is the sulphur atom of an interactive cysteine–histidine system, which generally develops nucleophilic character by protonic dissociation across $pK_a$ 3-4. A plausible component of this interactive system is the $-S^-/\cdot$ImH$^+$ ion-pair. Kinetic study of the reactions of a number of cysteine-proteinase catalytic-site thiols with groups 2,2'-dipyridyl disulphide (I) and with n-propyl 2-pyridyl disulphide (II) has demonstrated the value of 2-pyridyl disulphides as probes of the effects of microenvironment on catalytic-site characteristics (see Brocklehurst, 1982, and the

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1. 2-(N'-Acetyl-L-phenylalanylamino)ethyl 2'-pyridyl disulphide [compound (III)] and 2-(acetamido)ethyl 2'-pyridyl disulphide [compound (IV)] were synthesized by acylation of the common intermediate, 2-aminoethyl 2'-pyridyl disulphide, to provide examples of chromogenic thiol-specific substrate-derived two-protonic-state electrophilic probe reagents. These two reagents, together with n-propyl 2-pyridyl disulphide [compound (II)], provide structural variation in the non-pyridyl part of the molecule from a simple hydrocarbon side chain in compound (II) to a $P_1-P_2$ amide bond in compound (IV) and further to both a $P_1-P_2$ amide bond and a hydrophobic side chain (of phenylalanine) at $P_2$ as a potential occupant of $S_2$ subsites. 2. These disulphides were used as reactivity probes to investigate specificity and binding-site–catalytic-site signalling in a number of cysteine proteinases by determining (a) the reactivity at pH 6.0 at 25 °C at $I$ 0.1 of compound (III) (a close analogue of a good papain substrate) towards 2-mercaptoethanol, benzimidazol-2-ylmethanethiol [compound (V), as a minimal catalytic-site model], chymopapains $B_1-B_2$, chymopapain A, papaya proteinase $Q$, actinidin, cathepsin B and papain, (b) the effect of changing the structure of the probe as indicated above on the reactivities of compound (V) and of the last five of these enzymes, and (c) the forms of pH-dependence of the reactivities of papain and actinidin towards compound (III). 3. The kinetic data suggest that reagents of the type investigated may be sensitive probes of molecular recognition features in this family of enzymes and are capable not only of detecting differences in binding ability of the various enzymes but also of identifying enzyme–ligand contacts that provide for binding-site–catalytic-site signalling mechanisms. 4. The particular value of this class of probe appears to derive from the possibility of activating the 2-mercaptopyridine leaving group not only by formal protonation, as was recognized previously [see Brocklehurst (1982) Methods Enzymol. 87C, 427–469], but also by hydrogen-bonding to the pyridyl nitrogen atom when the appropriate geometry in the catalytic site is provided by enzyme–ligand contacts involving the non-pyridyl part of the molecule. The result of such contacts, as in the reaction of papain with compound (III), is a striking change in the shape of the pH–$k$ profile, which then resembles that of a pH–$k_{cat.}/K_m$ profile for substrate hydrolysis and is considered to derive from induction of a transition state geometry in which nucleophilic attack by the $S^-$ component of the catalytic site is assisted in neutral media by general acid-catalysed expulsion of the 2-mercaptopyridine leaving group of the probe, provided by the catalytic-site imidazolium ion.

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references cited above). 2-Pyridyl disulphides have previously been shown to possess two distinct characteristics that make them particularly valuable as thiol titrants and as environmentally sensitive reactivity probes. One, discovered by Grassetti & Murray (1967), derives from the existence of the 2-mercaptopyrindine product of a thiol–disulphide interchange largely as a thione with a high \( pK_a \) (approx. 9.9; Albert, 1963; Brocklehurst & Little, 1973) [eqns. (1) and (2)]:

\[
\begin{align*}
R-S-S-N^+ + R'SH & \rightleftharpoons R-S-S-R' + S-N^- + H^+ \\
\text{(1)}
\end{align*}
\]

\[
\begin{align*}
\text{N} & \quad \text{p}K_a = 9.9 \\
\text{S} & \quad + H^+ \\
\text{(2)}
\end{align*}
\]

The thione structure provides for (i) such a large value of the equilibrium constant for the reaction given in eqn. (1) that pyridine-2-thione formation is essentially stoichiometric with thiol when [pyridyl disulphide] \( \geq \) [thiol] and (ii) a convenient means of monitoring reaction by spectral analysis. The fact that the \( pK_a \) of 9.9 is not substantially lower than the \( pK_a \) values that characterize thiol groups (usually \( \leq 10 \)) accounts for the fact that the equilibrium constant for eqn. (1) does not decrease in acidic media as it does for analogous interchanges with non-pyridyl disulphides such as 5,5'-dithiobis-(2-nitrobenzoate), with consequent loss of product stoichiometry (Little & Brocklehurst, 1972). The other valuable characteristic of 2-pyridyl disulphides, reported by Brocklehurst & Little (1970, 1972, 1973), is the substantial increase in electrophilicity that accompanies the protonation of the ring nitrogen atom (\( pK_a \) approx. 2.5) [eqn. (3)]:

\[
\begin{align*}
\text{R-S-S-N}^+ & \quad \text{p}K_a \geq 2.5 \\
\text{N} & \quad \text{R-S-S-N}^- + H^+ \\
\text{(3)}
\end{align*}
\]

Since nucleophilic character in cysteine-proteinase catalytic sites is commonly generated by protic dissociation of \( pK_a \) 3–4, the coexistence of both \( -S^- / -\text{Im}H^+ \) ion-pair and the more electrophilic form of the probe, \( R-S-S-2-PyH^+ \), is maximized at \( \text{pH} \) values around 3–4 and profiles of \( \text{pH} \) versus second-order rate constant \( (k) \) for reactions of compound (I) and of compound (II) usually contain a striking rate maximum in this \( \text{pH} \) region, and provide thermodynamic and kinetic data about the interactive catalytic-site system of the enzyme and its reactivity towards both electrophilic forms of the probe molecule (see Fig. 1). In addition to their value as reactivity probes, the rapid reactions of compounds (I) and (II) with catalytically active cysteine proteinases at \( \text{pH} \) 3–4, with stoichiometric release of the chromophoric pyridine-2-thione (\( \lambda_{\text{max}} 343 \text{ nm} \)), provides a spectrophotometric titration of cysteine-proteinase catalytic sites (Brocklehurst & Little, 1973). Because non-interactive thiol groups often have \( pK_a \) values in the range 8–10, and thus exist almost wholly as the undissociated and essentially non-nucleophilic (R'SH) forms at \( \text{pH} \) 3–4, the titration of catalytic sites succeeds even in the presence of substantial quantities of low-\( M_r \) mercaptan and discriminates between active enzyme and denatured but still thiol-containing protein. The relatively high reactivity of the 2,2'-dipyridyl disulphide monoclonation towards the catalytic site of cathepsin \( \text{H} \), which exhibits an unusually low reactivity towards all electrophilic reagents tested thus far, currently provides the only satisfactory active-site titration for this enzyme (Willenbrock & Brocklehurst, 1985a).

In the present paper we report a new application for unsymmetrical 2-pyridyl disulphides as sensitive probes of cysteine-proteinase specificity and evidence for a new, if predictable, property of 2-pyridyl disulphides (activation by hydrogen-bonding to the ring nitrogen atom) that promises to facilitate the characterization of binding-site–catalytic-site signalling mechanisms. To these ends, this paper reports the synthesis of 2-(N'-acetyl-l-phenylalanylamino)ethyl 2'-pyridyl disulphide (III) and of 2-(acetamido)ethyl 2'-pyridyl disulphide (IV), as examples of substrate-derived 2-pyridyl disulphide probes, and kinetic studies of their reactions with a range of cysteine proteinases.
Materials and Methods

Enzymes

The isolation procedures for most of the enzymes have been described previously: papain (Baines & Brocklehurst, 1979); actinidin (Brocklehurst et al., 1981b); papaya proteinase Ω (papaya peptidase A) (Baines & Brocklehurst, 1982; Brocklehurst & Salih, 1983); bovine spleen cathepsin B (Willenbrock & Brocklehurst, 1984); chymopapain A (Baines et al., 1986). Chymopapains B1–B3 were isolated from the dried latex of Carica papaya supplied by Powell & Scholefield, Liverpool, U.K., by ion-exchange chromatography as described for the enzymes of fresh non-fruit papaya latex by Brocklehurst et al. (1985b). These three enzymes are eluted in the ion-exchange profile after chymopapain A and before papaya proteinase Ω, chymopapain B1 being the least basic and chymopapain B3 the most basic. Fully active forms of all of these enzymes containing one intact catalytic site per molecule are prepared by using covalent chromatography by thiol–disulphide interchange (Brocklehurst, et al., 1985a).

Reactivity probes

n-Propyl 2-pyrindyl disulphide was prepared from pyridine-2-thione and n-propanesulphenyl chloride and characterized as described by Shipton & Brocklehurst (1978).

Both 2-(N'-acetyl-L-phenylalanylamino)ethyl 2-pyrindyl disulphide (III) and 2-(acetamido)ethyl 2-pyrindyl disulphide (IV) were synthesized by acylation of 2-aminoethyl 2-pyrindyl disulphide. The dihydrochloride of this compound was prepared by oxidation of cysteamine by H2O2 to the thiosulphonate followed by reaction with pyridine-2-thione as described by Stuchbury et al. (1975). Before a particular acylation process, it was converted into the free base as follows. A solution of the dihydrochloride (1.13 g, 4.4 mmol) in water (10 ml) was adjusted to pH 10 with aq. NaOH, and the free base was extracted immediately into chloroform (3 × 10 ml). The combined chloroform extracts were dried over anhydrous Na2SO4 and, after removal of the drying agent by filtration, the filtrate was used to prepare acylated derivatives as described below.

A chloroform solution of 2-aminoethyl 2-pyrindyl disulphide free base (3 mmol) was cooled at 0 °C and N-t-butylxycarbonyl-L-phenylalanine N-hydroxysuccinimide ester (Sigma Chemical Co.) (1 g, 3.8 mmol) was added. The mixture was stirred at 0 °C for 2 h and was then washed sequentially, in a separating funnel, with 0.5 x equal volumes of aq. 1 m-citric acid, saturated aq. NaHCO3 and water. The chloroform layer was dried over anhydrous Na2SO4. Filtration and evaporation of the solvent gave 2-(N-t-butylxycarbonyl-L-phenylalanylamino)ethyl 2-pyrindyl disulphide as a colourless oil. The t-butylxycarbonyl group was removed by treatment with anhydrous formic acid (20 ml) at room temperature (approx. 22 °C) for 12 h. Formic acid was removed by rotary evaporation, the last traces being removed by trituration with diethyl ether (3 × 50 ml portions) and rotary evaporation. The ether-insoluble material was suspended in methylene chloride (20 ml) and treated sequentially with triethylamine (0.84 ml, 6 mmol) and acetic anhydride (0.4 ml, 4 mmol). After 15 min at room temperature, the mixture was washed firstly with water and then with saturated aq. NaHCO3, and was then dried over anhydrous Na2SO4.

Filtration and evaporation gave a crude preparation of compound (III) as a pale-green 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 ethyl acetate as eluent gave chromatographically homogeneous material (0.83 g, 74% yield), which was crystallized by dissolution in ethyl acetate (10 ml), followed by addition of hexane to turbidity, and storage at 4 °C. The crystalline material had m.p. 123 °C and [α]D +7.0 ± 0.5° (c 0.43 in methanol). (Found: C, 57.3; H, 5.7; N, 11.2; C11H12N2O2 requires C, 57.6; H, 5.6; N, 11.2%. δ N.m.r. data: δ (p.p.m.) (1H, tetramethylsilane); 1.7 (3H, s, CH3), 2.5–3.0 [4H, overlapping d–i, 2–CH2–], 3.4 (2H, q, CH2), 4.4 (1H, multiplet, CH), 7–8.5 (11H, complex multiplets, aromatic H, NH); the mass spectrum gave m/z 376 ([M+H]+; C17H18N2O2 requires M+ 375).

Acetylation of 2-aminoethyl 2-pyrindyl disulphide and purification of the product by column chromatography by a procedure analogous to that described above provided compound (IV) as a clear yellow oil, which 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 m−1 cm−1; Stuchbury et al., 1975).

Minimal catalytic-site model

Benzimidazol-2-ylmethanethiol was synthesized from o-phenylene diamine and thioglycollic acid essentially as described by Milner et al. (1964). It had m.p. 156–158 °C, and its hydrochloride had m.p. 218–220 °C, in agreement with the literature value.

Physical methods used in characterization of disulphide probes

These determinations, namely n.m.r. (Perkin–Elmer R32) and polarimetry (AA-5 polarimeter), were carried out in the Department of Chemistry, University of Salford.

Kinetics

All reactions were studied at 25.0 °C and I 0.1 in solutions containing 1 mm-EDTA with Dionex stopped-flow equipment at 343 nm (see Stuchbury et al., 1975). Buffer solutions were prepared double strength such that dilution 1 in 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), KH2PO4/NaOH (pH 5.8–7.8), Tris/HCl (pH 8.0–8.6), H3BO3/KCl/NaOH (pH 8.2–8.6), Na2P2O7/HCl (pH 8.0–9.4), glycine/NaOH (pH 9.0–10.2) and NaHCO3/NaOH (pH 9.0–10.6). The particularly rapid reactions of compound (III) with papain and with chymopapain B3 were studied under equimolar second-order conditions (3 μM). All other reactions were studied under pseudo-first-order conditions with [disulphide] [thiol]. Linear increase in
pseudo-first-order rate constant \( (k_{\text{obs}}) \) with increase in [disulphide] confirmed that under the conditions of concentration used ([disulphide] \( \leq 80 \mu M \)) the reactions obeyed overall second-order kinetics.

RESULTS AND DISCUSSION

Probe design and synthesis

We considered that investigation of the extended binding areas of cysteine proteinases and of the coupling of inhibitor binding (and hence, by analogy, of substrate binding) with catalytic-site chemistry would be aided by application of a series of thiol-specific chromogenic two-protonic-state reactivity probes each containing a 2-mercaptopyridine leaving group and a selection of ligands designed to engage selectively postulated parts of the binding area of a given enzyme. The interaction of proteinases with oligopeptide substrates and inhibitors is generally discussed in terms of the P-S notation introduced by Schechter & Berger (1967, 1978) (see Berger & Schechter, 1970) with due regard for the possibility of a continuum of flexibility in the extended binding areas of the enzymes (Fruton, 1977, 1982) and of the consequences of this for variation and degree of fit in the assumed P-S complementarities. As a prototype of such substrate-derived reactivity probes, we decided to synthesize the N-acetyl-L-phenylalanine derivative (III), to meet the known requirements in good substrates for papain of a hydrophobic side chain at P2 and an amide bond between P1 and P2 (Schechter & Berger, 1967, 1968; Berger & Schechter, 1970; Drenth et al., 1976; Lowe & Yuthavong, 1971a,b; Lowe, 1976). The presence of the second methylene group, by which the probe structure deviates from exact analogy with glycine at P1, which was dictated by the synthetic route, was not considered to be a serious drawback in view of the growing awareness of the permitted flexibility in P-S interaction discussed above. In addition, compound (IV) was synthesized to provide a probe containing the P1–P2 amide bond but without the hydrophobic side chain at P2.

n-Propyl 2-pyridyl disulphide (II) was already available in the laboratory and was used as a reference compound. The synthesis of both compounds (III) and (IV) involved the acylation of 2-aminoethyl 2'-pyridyl disulphide, the latter by using acetic anhydride and the former by using

![Scheme 1](image)

Scheme 1. Synthesis of 2-(N'-acetyl-L-phenylalanylamino)ethyl 2'-pyridyl disulphide (III) and 2-(acetamido)ethyl 2'-pyridyl disulphide

For experimental details see the text. Abbreviation: Boc, t-butyloxycarbonyl.
N-t-butyloxycarbonyl-L-phenylalanine hydroxysuccinimide ester, followed by removal of the t-butyloxycarbonyl group by formic acid treatment and subsequent acetylation of the freed terminal amino group by acetic anhydride (Scheme 1).

**Reactivity-probe kinetics**

The reactivity-probe studies reported below fall into three categories: (i) a comparison of the reactivities of the close analogue of a good papain substrate [compound (III)] towards eight cysteine proteinases, (ii) an examination of the effect of changing the probe structure, from one containing a simple alky1 side chain [compound (II)] to one containing a \( P_1-P_2 \) amide bond [compound (IV)], and then to the close analogue of a papain substrate containing both the \( P_1-P_2 \) amide bond and the hydrophobic side chain of L-phenylalanine to provide a significant \( P_2-S_2 \) interaction, on reactivity towards five of these enzymes, and (iii) a comparison of the pH-dependences of the reactivities of papain and actinidin towards compound (III).

**Variation in cysteine-proteinase specificity demonstrated by reactivities towards the papain substrate analogue [compound (III)].** The second-order rate constants for the reactions at pH 6.0 of compound (III) with the catalytic-site thiol groups of eight cysteine proteinases and with the thiol groups of two low-M_r mercaptans are presented in Table 1. The rate constants vary over a range of almost \( 1 \times 10^{7} \) to \( 3 \times 10^{8} \); the ratio of the largest value of \( k \) to the smallest being \( 3 \times 10^{6} \).

The lowest reaction is that of the simple low-M_r mercaptan, 2-mercaptoethanol \( (k = 3 \text{ M}^{-1} \text{s}^{-1}) \); at pH 6 the mercaptan \( (pK_a 9.6) \) exists very largely in the unreactive RSH form. The other low-M_r mercaptan, benzimidazol-2-ylmethanethiol (V), reacts about 200 times faster at pH 6 than 2-mercaptoethanol, the higher reactivity being attributed to the presence of substantial amounts of the nucleophilic ion-pair form of the mercaptan at this pH (see Brocklehurst, 1982).

Compound (V) may be regarded as a minimal model for a cysteine proteinase catalytic site in that substantial amounts of nucleophilic ion-pair exist in weakly acidic media. In the enzymes the reactivity of the catalytic-site ion-pair might be expected to be modulated by binding interactions between the subsites in the extended binding areas and suitable substituents in the non-pyridyl part of the disulphide probe molecule. The simplest way in which this might happen is by increasing the concentration of enzyme-probe adsorptive complex through which such chemical modification reactions might reasonably be expected to proceed (Brocklehurst & Dixon, 1976, 1977; Knowles, 1976). Even when a degree of saturation of enzyme (E) by probe reagent (R) cannot be detected, by deviation from second-order kinetics, it may be inappropriate to ignore the possibility of complex formation before covalency change [eqn. (4)]:

\[
E + R \xrightarrow{k_{-1}} ER \xrightarrow{k_{+2}} \text{products}
\]

When a chemical modification reaction with [R] \( \gg [E] \) obeys (pseudo)-first-order kinetics with respect to time and second-order kinetics with respect to concentration, the steady-state expression [eqn. (5)] is, for practical purposes, a valid expression for the apparent second-order rate constant, \( k \) (Shipton & Brocklehurst, 1977; Cornish-Bowden, 1979), which simplifies to eqn. (6) when the quasi-equilibrium condition may legitimately be made:

\[
k = k_{+2}/k_{-1} + k_{+2}
\]

\[
k = k_{+2}/k_r
\]

where \( k_r = k_{-1}/k_{+2} \).

The conventional condition for quasi-equilibrium around the postulated ER complex \( (k_{+2} \ll k_{-1}) \) is a necessary consequence of the condition \( k \ll k_{+2} \) (Brocklehurst, 1979), and a reasonable lower limit of \( k_{+2} \) for association of enzyme molecules with small ligands may well be about \( 1 \times 10^{7} \text{ M}^{-1} \text{s}^{-1} \). Thus in terms of eqn. (6) a change in structure of probe or enzyme that results in a decrease in the dissociation constant, \( k_r \), would produce an increase in apparent second-order rate constant, \( k \). The reactions listed in Table 1 obeyed good second-order kinetics under the conditions of concentration given in the Materials and methods section, and it seems reasonable to consider the apparent second-order rate constant as the ratio \( k_{+2}/k_r \) given in eqn. (6). The other possible kinetic consequence of a change in structure in either component of the reactant system is a change in \( k_{+2} \), which may reflect change in transition state geometry. In terms of eqn. (6) changes in either \( k_{+2} \) or \( k_r \) are observed as changes in \( k \). For the reaction of probe (III) with chymopapain A, the value of \( k \) is similar to that for the analogous reaction with the minimal catalytic-site model, benzimidazol-2-ylmethanethiol, and thus the non-pyridyl part of the probe presumably does not make significant binding contacts with this enzyme. The other enzymes listed in Table 1 do appear to make kinetically significant binding contacts with the probe and to considerably different extents. The rate-enhancement factors that compare the reactivity of a given enzyme to the reactivity of the minimal catalytic-site model cover the range 30 for chymopapain B, 40 for papaya proteinase \( \Omega \), 50 for chymopapain B, 200 for actinidin, \( 10^4 \) for cathepsin B, and \( 10^6 \) for papain and for chymopapain B. It is perhaps not surprising that a large rate-enhancement factor is observed for the papain reaction in view of the fact that the probe reagent (III) was designed to suit the known binding requirements of this enzyme. The wide range of values of the rate-enhancement factor for the various cysteine proteinases and the good separation between all pairs of values seem particularly valuable because these suggest that reagents of this type may well be very sensitive probes of molecular recognition features in this family of enzymes.

The possibility that reactivity probes of this type may be capable not only of detecting differences in binding ability of the various enzymes (effects on \( k_r \)) but also of identifying enzyme-ligand contacts that provide for
Table 1. Second-order rate constants ($k$) for the reactions of 2-(N′-acetyl-L-phenylalanylamino)ethyl 2′-pyridyl disulphide with the catalytic-site thiol groups of some cysteine proteinases, and with low-$M_f$ mercaptans, at pH 6.0 at 25°C at $I$ 0.1

<table>
<thead>
<tr>
<th>Location of thiol group*</th>
<th>Rate-enhancement factor</th>
<th>$k$ (M$^{-1}$ s$^{-1}$)</th>
<th>[k (enzyme)]/[k (MCM)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Mercaptoethanol</td>
<td></td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>Benzimidazol-2-ylmethanethiol (minimal catalytic-site model, MCM)</td>
<td></td>
<td>5.0 x 10$^4$</td>
<td>–</td>
</tr>
<tr>
<td>Chymopapain A</td>
<td></td>
<td>7.0 x 10$^4$</td>
<td>1.4</td>
</tr>
<tr>
<td>Chymopapain B$_{1}$</td>
<td></td>
<td>1.6 x 10$^4$</td>
<td>32</td>
</tr>
<tr>
<td>Papaya protease $\Omega$</td>
<td></td>
<td>1.9 x 10$^4$</td>
<td>38</td>
</tr>
<tr>
<td>Chymopapain B$_{2}$</td>
<td></td>
<td>2.7 x 10$^4$</td>
<td>54</td>
</tr>
<tr>
<td>Actinidin</td>
<td></td>
<td>1.1 x 10$^6$</td>
<td>220</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td></td>
<td>6.0 x 10$^6$</td>
<td>1200</td>
</tr>
<tr>
<td>Papain</td>
<td></td>
<td>4.5 x 10$^6$</td>
<td>9000</td>
</tr>
<tr>
<td>Chymopapain B$_{3}$</td>
<td></td>
<td>9.0 x 10$^6$</td>
<td>18000</td>
</tr>
</tbody>
</table>

* For all of the enzymes, the thiol group is part of the catalytic site; each of the chymopapains contains another thiol group, which reacts much more slowly.

binding-site–catalytic-site signalling mechanisms (effects on $k_{on}$) is discussed below when the pH-dependences of the reactions of papain and actinidin with compound (III) are considered.

Effect of variation of probe structure. Table 2 contains the results of a study for benzimidazol-2-ylmethanethiol and for five cysteine proteinases of varying the structure of the reactivity probe so as to vary the opportunities for binding contacts. Thus probe (II), n-propyl 2-pyridyl disulphide, contains only a short hydrocarbon side chain, probe (IV) contains the P$_1$–P$_2$ amide bond but no potential occupant for the hydrophobic S$_h$ binding pocket, and probe (III) contains both the P$_1$–P$_2$ amide bond and the phenylalanine side chain at P$_2$. The responses of the thiol-containing reactant molecules (enzymes and minimal catalytic-site model) to structural change in the disulphide probe are of four types, designated (a)–(d) in Table 2.

(a) Reaction of the minimal catalytic-site model is insensitive to change in probe structure, as would be predicted because of the lack of binding interactions for the low-$M_f$ reactant system and because the variable part of the probe structure is insulated electronically from the electrophilic centre of the disulphide bond by two methylene groups.

(b) The reactivities of both papain and cathepsin B are substantially increased by the presence of the P$_1$–P$_2$ amide bond and are further substantially increased (both times by about an order of magnitude) by the addition of the phenylalanine side chain at P$_2$. Presumably, for these two enzymes, both structural features constitute important specificity sites, as had been deduced previously for papain from steady-state kinetic studies of substrate hydrolysis (see Lowe, 1976) and from X-ray crystallographic data (Drenth et al., 1976).

(c) The reactivities of both actinidin and papaya protease $\Omega$ are insensitive to incorporation of the P$_1$–P$_2$ amide bond into the probe molecule but are increased substantially consequent on the additional incorporation of the hydrophobic phenylalanine side chain at P$_2$. When both specificity features are present in the probe molecule, the reactivity of papain is respectively 40 times and 250 times greater than the reactivities of actinidin and papaya protease $\Omega$. Papain is 50 times more reactive towards the probe containing only the P$_1$–P$_2$ amide bond than actinidin but is now only 40 times more reactive than papaya protease $\Omega$. When the probe contains only the n-propyl side chain, the advantage displayed by papain is even less, its reactivity being only 7 times greater than that of actinidin and 4 times greater than that of papaya protease $\Omega$. The substantially greater kinetic specificity displayed by papain than by actinidin towards the phenylalanine derivative (III) is in accord with the main structural difference between the active-centre regions of the two enzymes deduced from the crystallographic data (Drenth et al., 1976) and is in accord with the differences in values of $k_{cat}$/[$K_m$ for substrates bearing aromatic N-substituents, as against those devoid of such substituents, where the values of the specificity constant are 10–100 times lower (Baker et al., 1980). Actinidin differs from papain in a number of respects in the S$_h$-subsite region: Trp-69 $\rightarrow$ Tyr-9, Pro-68 $\rightarrow$ Ile-70, Phe-207 $\rightarrow$ Ser-213 and Val-133 $\rightarrow$ Ala-136. In particular, Ser-205, the residue at the end of the S$_h$ subsite in papain, is Met-211 in actinidin, which makes the pocket significantly shorter and may account in part for the differences in specificity discussed above. The still relatively large difference in reactivity between papain and actinidin (a factor of 50-fold) towards the probe that lacks the phenylalanine side chain but contains the P$_1$–P$_2$ amide bond is somewhat surprising in view of the close similarity in structure between the two enzymes in the region more immediately close to the catalytic-site region. Similar differences in reactivity and hence kinetic specificity between papain and papaya protease $\Omega$ are apparent from the data in Table 2. Little structural information about papaya protease $\Omega$ is available, but differences between the specificity of this enzyme and that of papain have been deduced from inhibitor-binding studies (Schack & Kaarsholm, 1984).

(d) The reactivity of chymopapain A towards all three probes differs little from the reactivity of the minimal catalytic-site model, compound (V). Inclusion of the P$_1$–P$_2$ amide bond actually results in a small decrease in reactivity towards chymopapain A that is not changed significantly by inclusion, additionally, of the phenylalanine side chain at P$_2$. Clearly the binding interactions

1987
Specificity probes for cysteine proteinases

Vol. 244

on which the kinetic specificity of chymopapain A depends have not been defined by the present study. Whether the active centre of this enzyme is 'featureless', as was suggested for papaya proteinase Ω by Schack & Kaarsholm (1984), or whether different interactions in or around the S₁ and S₂ subsites from those provided by the three probes used in this work are required, remains to be determined.

pH-dependences of the reaction of the papain-substrate-analogue probe (III) with papain and with actinidin. The pH-dependence of the second-order rate constants (k) for these two reactions are shown in Fig. 2. These pH-k profiles are remarkable in two respects, in addition to the substantial increases in reactivity at pH 6 produced by incorporating the P₁–P₂ disulphide bond and the phenylalanine side chain at P₂ into the probe structure, which were discussed in connection with the data in Table 2.

Vol. 244
Fig. 2. pH-dependence of the second-order rate constant for reactions of 2-(\(N^\prime\)-acetyl-L-phenylalanylamino)ethyl 2'-pyridyl disulphide (III) with (a) papain and (b) actinidin at 25 °C at \(10^{-1} \) in aqueous buffers

The points are experimental for the reaction with papain and for the reaction with actinidin, and for the papain reaction the line is theoretical for:

\[
k = \frac{k}{[1 + (10^{\text{pH}+10})(10^pK_1) + (10^{\text{pH}})(10^pK_1)]}
\]

where

\[
k = 4.6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}, \ pK_1 = 3.9 \text{ and } pK_{II} = 9.3.
\]

Firstly, the shape of the pH–k profile for the reaction of papain with compound (III) (Fig. 2) is fundamentally different from the shape of the profile for the reaction of papain with n-propyl 2-pyridyl disulphide (Fig. 1) and is similar to profiles of pH versus \(k_{\text{cat}}/K_m\) for papain-catalysed hydrolysis (see, e.g., Lowe, 1976; Brocklehurst et al., 1981a). Secondly, the marked change in profile shape found for the papain reaction is not found also for the corresponding reaction of actinidin.

The very marked change in the shape of the pH–k profile for the papain reaction consequent upon incorporation of specific binding features into the probe molecule suggests that substrate-derived two-protonic-state electrophiles of the R–S–S–2-Py type might provide a sensitive method for the investigation of binding-site–catalytic-site signalling mechanisms. As pointed out in the Introduction, the rate maximum at pH values around 3–4 (Fig. 1) found for reactions for several cysteine proteinases, including papain and actinidin, with featureless probes such as n-propyl 2-pyridyl disulphide is considered to arise from the coexistence of significant concentrations of the probe cation (R–S–S–2-PyH\(^+\)) and the nucleophilic ion-pair (–S\(^–\)/–ImH\(^+\)). The results in Fig. 2 suggest that, if suitable binding interactions take place between the probe molecule and the extended binding site of the enzyme, a transition-state geometry like that depicted schematically in (VI) may be achieved such that both partners of the –S\(^–\)/–ImH\(^+\) ion-pair react synchronously, as nucleophile and general acid respectively, with the neutral (unprotonated) form of the probe molecule.

The high reactivity of papain towards the substrate-analogue probe (III) at pH 6 is considered to arise, therefore, not only as a consequence of better binding of the probe molecule [i.e., a low \(K_r\), in eqn. (6)] when it contains the P1–P2 amide bond and the phenylalanine side chain at P2, but also because the effects of binding interactions are reflected in a new transition-state geometry (VI) in which hydrogen-bonding of the histidine side chain (–ImH\(^+\)) with the nitrogen atom of the leaving group provides for general acid catalysis with consequent increase in \(k_{12}\) [eqn. (6)]. The latter contribution is clearly absent when the probe contains only the n-propyl side chain (Fig. 1) and activation of the probe is by formal protonation at lower pH values than by hydrogen-bonding with –ImH\(^+\) in approximately neutral media.

It is noteworthy that a striking rate maximum at pH values around 6 is not a feature also of the pH–k profile for the reaction of actinidin with probe (III) (Fig. 2) even though incorporation of the l-phenylalanine side chain into the probe structure does produce a 200-fold increase in rate at pH 6 (Table 2). It may be significant that no substantial increase in rate (Table 2) is provided in the actinidin reaction when the P1–P2 amide bond alone is incorporated into the probe structure. A suitable interaction of this amide bond may be necessary for rearrangement of transition-state geometry, either as such or as a transmission mechanism for the conformational consequences of P2–S2 binding.

These results suggest an explanation in molecular terms for the amplification mechanism provided by the analogous P1–P2 peptide bond in substrates for papain deduced by Lowe & Yuthavong (1971a,b) on the basis of results from steady-state kinetics and model building. It seems possible that specific interaction of this peptide bond with the enzyme backbone may permit or facilitate general acid catalysis of the expulsion of the leaving group in the acylation process of the catalytic act.

1987
General comments

Unsymmetrical 2-pyridyl disulphides have been used previously for purification of thiol-containing proteins by covalent chromatography (Brocklehurst et al., 1973, 1985a), as site-specific delivery vehicles for spectroscopic reporter groups (Stuchbury et al., 1975), as heterobi-functional cross-linking reagents (Carlsson et al., 1978) and as two-protonic-state electrophilic reactivity probes for characterization of interactive catalytic-site systems and their immediate microenvironments (Brocklehurst, 1982). The new application, here reported, derives from the possibility of activating the 2-mercaptopropiridine leaving group not only by formal protonation at low pH but also by its association with a hydrogen-bond donor when association is provided for by specific interactions involving the extended enzyme binding site and the non-pyrilid part of the probe. The existence of this additional mechanism for probe activation provides opportunities to define the nature of binding-site-catalytic-site signalling mechanisms, which might be an important facet of molecular recognition processes. The value of the cysteine proteinase family in providing considerable structural variation in and around the catalytic site, despite, in many cases, considerable sequence homology (Kamphuis et al., 1985), linked with evidence for considerable variation in catalytic-site chemistry (Brocklehurst, 1986), is now augmented by the demonstration in the present work of a wide variation in specificity characteristics and, at least in some cases, in binding-site-catalytic-site signalling mechanisms.

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