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

Activation of 2-pyridyl disulphides by hydrogen-bonding

Keith BROCKLEHURST,*‡ Devanand KOWLESSUR,* Mairead O'DRISCOLL,* Geeta PATEL,*§
Siobhan QUENBY,* Erdjan SALIH,*‖ William TEMPLETON,* Emrys W. THOMAS† and
Frances WILLENBROCK*¶
*Department of Biochemistry, Medical College of St. Bartholomew's Hospital (University of London), Charterhouse Square,
London EC1M 6BQ, U.K., and †Department of Biological Sciences, University of Salford, Salford M5 4WT, U.K.

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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_3, chymopapain A, papaya proteinase Ω, 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–$k_{\mathrm{cat.}}/K_{\mathrm{m}}$ 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.

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 $-S^-/-ImH^+$ ion-pair. Kinetic study of the reactions of a number of cysteine-proteinase catalytic-site thiol groups with 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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(I)

(II)
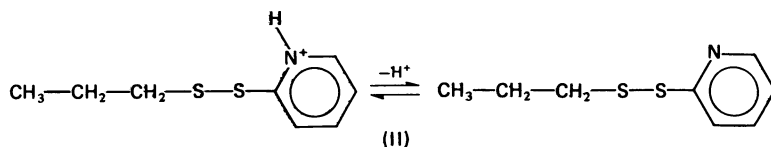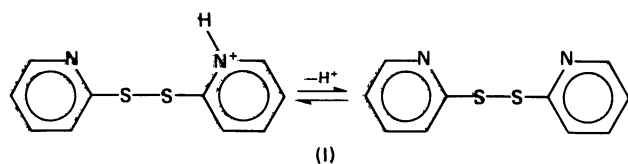
references cited above). 2-Pyridyl disulphides have previously been shown to possess two distinct characteristics that make them particularly valuable as thiol titrants and as environmentally sensitive reactivity probes. One, discovered by Grassetti & Murray (1967), derives from the existence of the 2-mercaptopyridine 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)]:

$$(1)$$

$$(2)$$
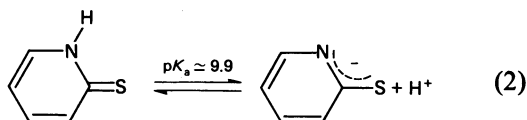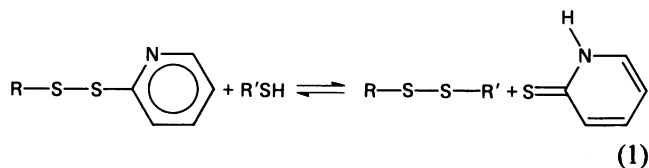
The thione structure provides for (i) such a large value of the equilibrium constant for the reaction given in eqn. (1) that pyridine-2-thione formation is essentially stoichiometric with thiol when [pyridyl disulphide] ⩾ [thiol] and (ii) a convenient means of monitoring reaction by spectral analysis. The fact that the pK_a of 9.9 is not substantially lower than the pK_a values that characterize thiol groups (usually < 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)]:

$$(3)$$
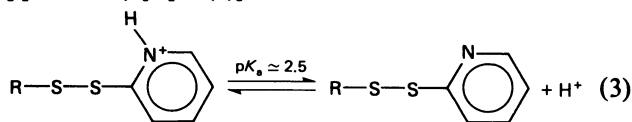
Since nucleophilic character in cysteine-proteinase catalytic sites is commonly generated by protonic dissociation of pK_a 3–4, the coexistence of both $-S^-/-ImH^+$ ion-pair and the more electrophilic form of the probe, R–S–S–2-PyH$^+$, is maximized at pH values around 3–4 and profiles of pH versus second-order rate constant (k) for reactions of compound (I) and of compound (II) usually

contain a striking rate maximum in this 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 pH 3–4, with stoichiometric release of the chromophoric pyridine-2-thione ($\lambda_{max.}$ 343 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 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 mono-cation towards the catalytic site of cathepsin 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.

(III)

(IV)
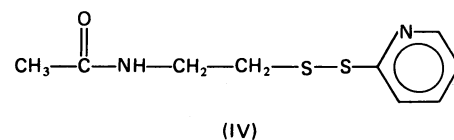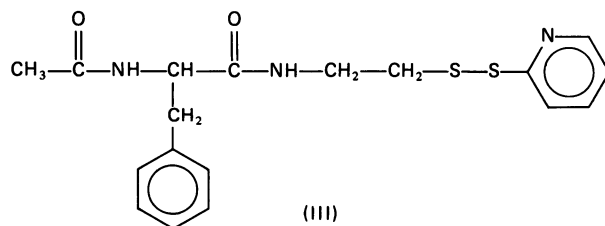
An additional application of compound (III) is that it can be used as a rapid active-centre titrant for most of the cysteine proteinases evaluated, at pH values around 6, even in the presence of extraneous non-catalytic-site thiol groups.

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 B_1–B_3 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 B_1 being the least basic and chymopapain B_3 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-pyridyl 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'-pyridyl disulphide (III) and 2-(acetamido)ethyl 2'-pyridyl disulphide (IV) were synthesized by acylation of 2-aminoethyl 2'-pyridyl disulphide. The dihydrochloride of this compound was prepared by oxidation of cysteamine by H_2O_2 to the thiolsulphonate 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 Na_2SO_4, 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'-pyridyl disulphide free base (3 mmol) was cooled at 0 °C and N-t-butyloxycarbonyl-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 × equal volumes of aq. 0.1 M-citric acid, saturated aq. $NaHCO_3$ and water. The chloroform layer was dried over anhydrous Na_2SO_4. Filtration and evaporation of the solvent gave 2-(N-t-butyloxycarbonyl-L-phenylalanylamino)ethyl 2'-pyridyl disulphide as a colourless oil. The t-butyloxycarbonyl 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. $NaHCO_3$, and was then dried over anhydrous Na_2SO_4.

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 $[\alpha]_D^{20}$ +7.0±0.05° (c 0.43 in methanol). (Found: C, 57.3; H, 5.7; N, 11.2; $C_{17}H_{19}N_3O_2S_2$ requires C, 57.6; H, 5.6; N, 11.2%.) N.m.r. data: δ (p.p.m.) ([²H]dimethyl sulphoxide) 1.7 (3H, s, CH_3), 2.5–3.0 [4H, overlapping d+t, 2–CH_2–], 3.4 (2H, q, CH_2), 4.4 (1H, multiplet, CH), 7–8.5 (11H, complex multiplets, aromatic H, NH); the mass spectrum gave m/z 376 [(M^++1); $C_{17}H_{19}N_3O_2S_2$ requires M^+ 375].

Acetylation of 2-aminoethyl 2'-pyridyl 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 $M^{-1} \cdot cm^{-1}$; Stuchbury et al., 1975).

Minimal catalytic-site model

Benzimidazol-2-ylmethanethiol was synthesized from o-phenylenediamine 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), KH_2PO_4/NaOH (pH 5.8–7.8), Tris/HCl (pH 8.0–8.6), H_3BO_3/KCl/NaOH (pH 8.2–8.6), $Na_4P_2O_7$/HCl (pH 8.0–9.4), glycine/NaOH (pH 9.0–10.2) and $NaHCO_3$/NaOH (pH 9.0–10.6).

The particularly rapid reactions of compound (III) with papain and with chymopapain B_3 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 ($k_{obs.}$) with increase in [disulphide] confirmed that under the conditions of concentration used ([disulphide] $\leqslant 80\ \mu\text{M}$) 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 P_2 and an amide bond between P_1 and P_2 (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 P_1, 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 P_1–P_2 amide bond but without the hydrophobic side chain at P_2. 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
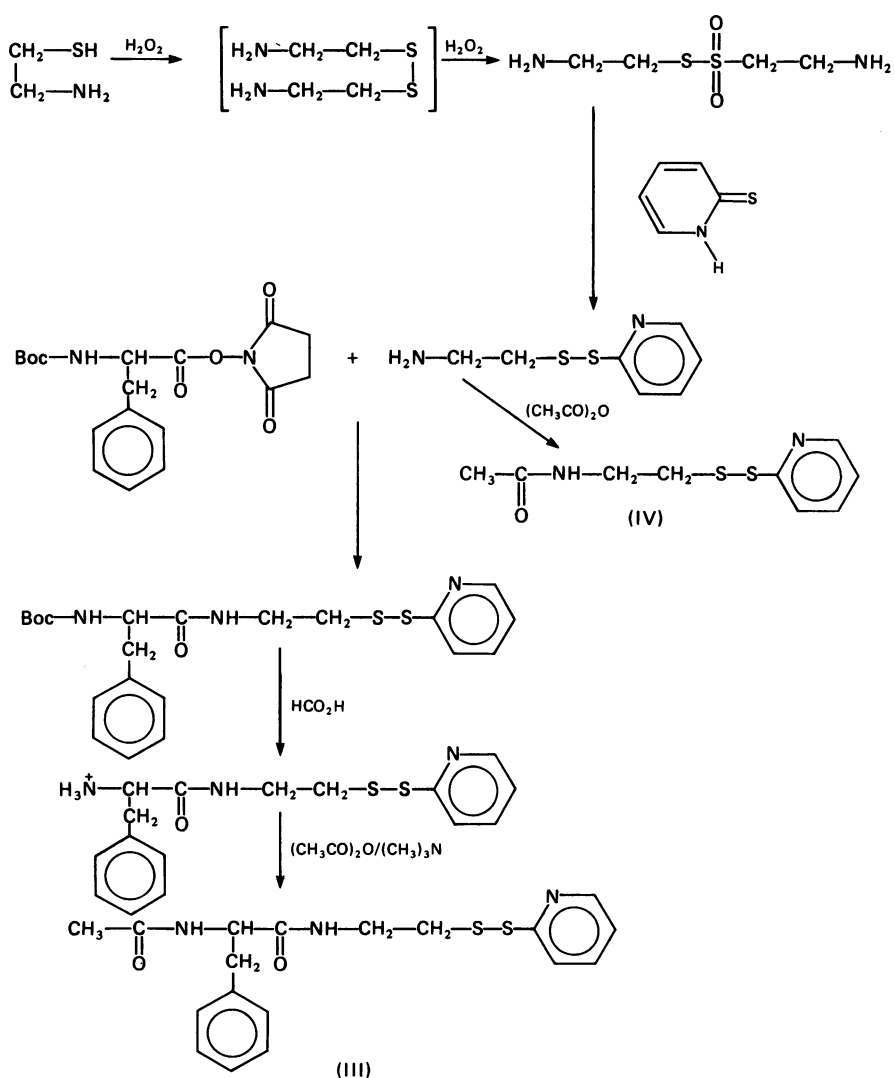
Scheme 1. Synthesis of 2-(N′-acetyl-L-phenylalanylamino)ethyl 2′-pyridyl disulphide (III) and 2-(acetamido)ethyl 2′-pyridyl disulphide

For experimental details see the text. Abbreviation: Boc, t-butyloxycarbonyl.

N-t-butyloxycarbonyl-L-phenylalanine hydroxysuccin-imide ester, followed by removal of the t-butyloxy-carbonyl 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 alkyl 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 1×10^7 $M^{-1} \cdot s^{-1}$, the ratio of the largest value of k to the smallest being 3×10^6.

The slowest reaction is that of the simple low-M_r mercaptan, 2-mercaptoethanol ($k = 3$ $M^{-1} \cdot s^{-1}$); 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)]:

$$E + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ER \xrightarrow{k_{+2}} products \qquad (4)$$
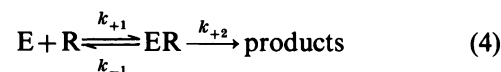
When a chemical modification reaction with [R] $\gg$ [E] obeys (pseudo)-first-order kinetics with respect to time and second-order kinetics with respect to concentration, the steady-state expression [eqn. (5)] is, for practical purposes, a valid expression for the apparent second-order rate constant, k (Shipton & Brocklehurst, 1977; Cornish-Bowden, 1979), which simplifies to eqn. (6) when the quasi-equilibrium condition may legitimately be made:

$$k = k_{+1}k_{+2}/(k_{-1}+k_{+2}) \qquad (5)$$
$$k = k_{+2}/K_r \qquad (6)$$

where

$$K_r = k_{-1}/k_{+1} \qquad (7)$$

The conventional condition for quasi-equilibrium around the postulated ER complex $(k_{+2} \ll k_{-1})$ is a necessary consequence of the condition $k \ll k_{+1}$ (Brocklehurst, 1979), and a reasonable lower limit of k_{+1} for association of enzyme molecules with small ligands may well be about 1×10^7–1×10^8 $M^{-1} \cdot s^{-1}$. 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_1, 40 for papaya proteinase Ω, 50 for chymopapain B_2, 200 for actinidin, 10^3 for cathepsin B, and 10^4 for papain and for chymopapain B_3. 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
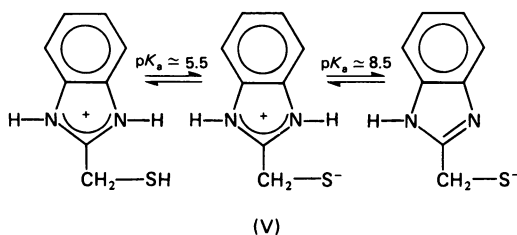
(V)

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_r mercaptans, at pH 6.0 at 25 °C at I 0.1

Location of thiol group*	k ($M^{-1} \cdot s^{-1}$)	Rate-enhancement factor [k (enzyme)/k (MCM)]
2-Mercaptoethanol	3	–
Benzimidazol-2-ylmethanethiol (minimal catalytic-site model, MCM)	5.0×10^2	–
Chymopapain A	7.0×10^2	1.4
Chymopapain B_1	1.6×10^4	32
Papaya proteinase Ω	1.9×10^4	38
Chymopapain B_2	2.7×10^4	54
Actinidin	1.1×10^5	220
Cathepsin B	6.0×10^5	1200
Papain	4.5×10^6	9000
Chymopapain B_3	9.0×10^6	18000

* 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_{+2}) 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_2 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_r 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 proteinase Ω 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 proteinase Ω. 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 proteinase Ω. 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 proteinase Ω. 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_2-subsite region: Trp-69 → Tyr-9, Pro-68 → Ile-70, Phe-207 → Ser-213 and Val-133 → Ala-136. In particular, Ser-205, the residue at the end of the S_3 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 calaytic-site region. Similar differences in reactivity and hence kinetic specificity between papain and papaya proteinase Ω are apparent from the data in Table 2. Little structural information about papaya proteinase Ω 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

Table 2. Differences in the response of some cysteine proteinases to change in probe structure in the P_1–P_2 region: second-order rate constants k_1–k_3 for the reactions with R–CH$_2$–CH$_2$–S–S–⊙ at pH 6.0 at 25 °C at I 0.1

Type	Response to change in probe structure	Location of thiol group	R = methyl (II) (k_1)	R = acetamido (IV) (k_2)	R = N-acetyl-phenylalanylamino (III) (k_3)	k_2/k_1	k_3/k_2	k_3/k_1	k_3 (papain)/k_3 (enzyme or model)	k_2 (papain)/k_2 (enzyme or model)	k_1 (papain)/k_1 (enzyme or model)
(a)	$k_1 \simeq k_2 \simeq k_3$	Benzimidazol-2-ylmethanethiol (minimal catalytic-site model with no binding interactions)	7.0×10^2	5.0×10^2	5.0×10^2	0.7	1.0	0.7	9×10^3	60	5
(b)	$k_1 \ll k_2 \ll k_3$	Papain	3.5×10^3	3.0×10^4	4.5×10^6	8.6	150	1300	1	1	1
		Cathepsin B	4.4×10^3	6.1×10^4	6.0×10^5	14	10	140	7.5	0.5	0.8
(c)	$k_1 \simeq k_2 \ll k_3$	Actinidin	5.0×10^2	5.8×10^2	1.1×10^5	1.2	190	220	40	52	7
		Papaya proteinase Ω	9.0×10^2	8.0×10^2	1.8×10^4	0.9	23	20	2.5×10^2	38	4
(d)	$k_1 \gg k_2 \simeq k_3$	Chymopapain A	2.0×10^3	8.4×10^2	7.0×10^2	0.4	0.8	0.4	2×10^4	36	2

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 S_1 and S_2 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 P_1–P_2 amide bond and the phenylalanine side chain at P_2 into the probe structure, which were discussed in connection with the data in Table 2.
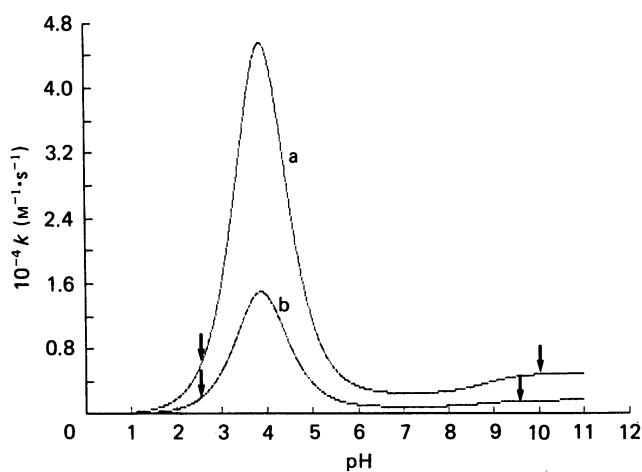
Fig. 1. pH-dependence of the second-order rate constant for reactions of papain and of actinidin with (a) n-propyl 2-pyridyl disulphide and (b) 2,2′-dipyridyl disulphide at 25 °C at I 0.1 in aqueous buffers

The lines are theoretical curves that gave good fits to the major features of experimental data for the reactions of papain reported by Shipton & Brocklehurst (1978); in curve b the additional sigmoid wave suggested by the tendency of the data to lie somewhat above the fitted line in the pH region 4.3–5.5 is not shown; the arrows indicate the pH ranges over which the data were collected. The curves were computed by using a BBC microcomputer and:

$$k = \hat{k}_1/[1 + (10^{-pH})(10^{pK_I}) + (10^{-pH})(10^{pK_{II}})(10^{pH})]$$
$$+ \hat{k}_2/[1 + 10^{-pH})(10^{pK_{II}}) + (10^{-pK_{III}})(10^{pH})]$$
$$+ \hat{k}_3/[1 + (10^{-pH})(10^{pK_{III}})]$$

where for curve a $\hat{k}_1 = 1.3 \times 10^5$ M^{-1}·s^{-1}, $\hat{k}_2 = 2.4 \times 10^3$ M^{-1}·s^{-1}, $\hat{k}_3 = 5.0 \times 10^3$ M^{-1}·s^{-1}, pK_I = 3.86, pK_{II} = 3.89 and pK_{III} = 8.8, and for curve b $\hat{k}_1 = 4.2 \times 10^4$ M^{-1}·s^{-1}, $\hat{k}_2 = 8.5 \times 10^2$ M^{-1}·s^{-1}, $\hat{k}_3 = 1.7 \times 10^3$ M^{-1}·s^{-1}, pK_I = 3.85, pK_{II} = 3.90 and pK_{III} = 8.8. The corresponding pH–k profiles for the analogous reactions of actinidin (not shown) are of similar form, with for the reaction with n-propyl 2-pyridyl disulphide $\hat{k}_1 = 6.7 \times 10^3$ M^{-1}·s^{-1}, $\hat{k}_2 = 5.0 \times 10^2$ M^{-1}·s^{-1}, $\hat{k}_3 = 1.15 \times 10^3$ M^{-1}·s^{-1}, pK_I = 2.7, pK_{II} = 3.15 and pK_{III} = 9.4, and for the reaction with 2,2′-dipyridyl disulphide $\hat{k}_1 = 2.3 \times 10^{-4}$ M^{-1}·s^{-1}, $\hat{k}_2 = 2.0 \times 10^3$ M^{-1}·s^{-1}, $\hat{k}_3 = 5.0 \times 10^3$ M^{-1}·s^{-1}, pK_I = 2.45, pK_{II} = 3.1 and pK_{III} = 10.0.
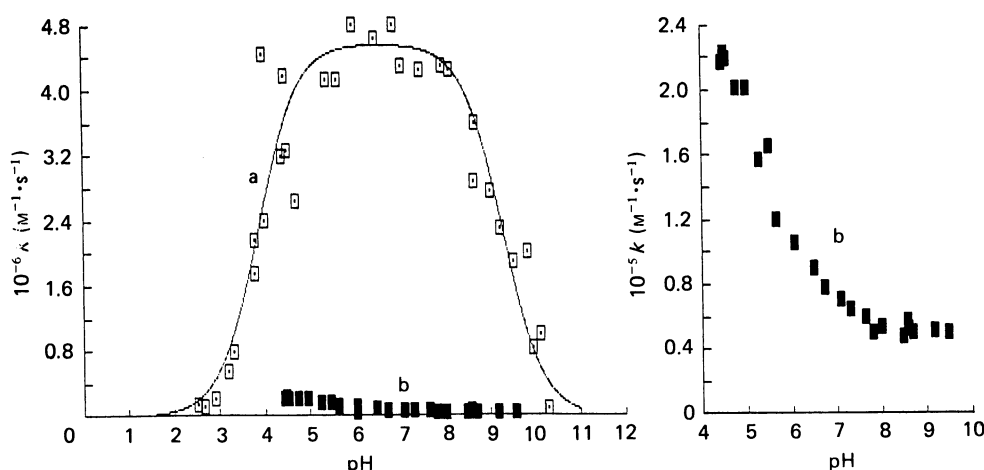
Fig. 2. pH-dependence of the second-order rate constant for reactions of 2-(N'-acetyl-L-phenylalanylamino)ethyl 2'-pyridyl disulphide (III) with (a) papain and (b) actinidin at 25 °C at I 0.1 in aqueous buffers

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:

$$k = \tilde{k}/[1 + (10^{-\text{pH}})(10^{pK_{\text{I}}}) + (10^{-pK_{\text{II}}})(10^{\text{pH}})]$$

where

$$k = 4.6 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}, \ pK_{\text{I}} = 3.9 \text{ and } pK_{\text{II}} = 9.3.$$

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 $k_{\text{cat.}}/K_{\text{m}}$ 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 synchro-

nously, 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 P_1–P_2 amide bond and the phenylalanine side chain at P_2, 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_{+2} [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 P_1–P_2 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 P_2–S_2 binding.

These results suggest an explanation in molecular terms for the amplification mechanism provided by the analogous P_1–P_2 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.

(VI)
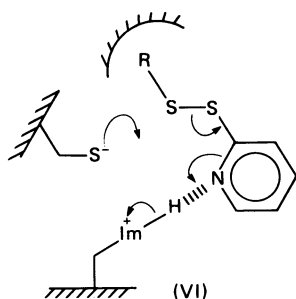
General comments

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 heterobifunctional 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-mercaptopyridine 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-pyridyl 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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