Evaluation of hydrogen-bonding and enantiomeric P₂–S₂ hydrophobic contacts in dynamic aspects of molecular recognition by papain

Manij PATEL,* Irfan S. KAYANI,* William TEMPLETON,* Geoffrey W. MELLOR,* Emrys W. THOMAS† and Keith BROCKLEHURST*‡

*Laboratory of Structural and Mechanistic Enzymology, Department of Biochemistry, Queen Mary and Westfield College, University of London, Mile End Road, London E1 4NS, U.K., and †Department of Biological Sciences, University of Salford, Salford M5 4JW, U.K.

1. 2-(*N*'-Acetyl-D-phenylalanyl)hydroxyethyl 2'-pyridyl disulphide (compound IV) {m.p. 59 °C; [α]_D²⁰ –6.6° (c 1.2 in methanol)} was synthesized. 2. The results of a study of the pH-dependence of the second-order rate constant (*k*) for its reaction with the catalytic-site thiol group (Cys-25) of papain (EC 3.4.22.2) together with analogous kinetic data for the reactions of related time-dependent inhibitors, notably the L-enantiomer of compound (IV) (compound III) and the L- and D-enantiomers of 2-(*N*'-acetylphenylalanyl)aminoethyl 2'-pyridyl disulphide (compounds I and II respectively), were used to assess the contributions of the (P₁)–NH...O=C < (Asp-158) and (P₂) > C=O...H–N–(Gly-66) hydrogen bonds and enantiomeric P₂–S₂ hydrophobic contacts in two manifestations of dynamic molecular recognition in papain–ligand association: (a) signalling to the catalytic-site region to provide for a (His-159)–Im⁺–H-assisted transition state and (b) the dependence of P₂–S₂ stereoselectivity on hydrogen-bonding interactions outside the S₂ subsite. The analysis involved determination of the reactivities of individual ionization states of the reactions (pH-independent rate constants, $\tilde{k}$) and associated macroscopic p*K*_a values and difference kinetic specificity energies ($\Delta\Delta G_{\text{KS}} = -RT\ln(\tilde{k}_1/\tilde{k}_2)$, where $\tilde{k}_1$ is the pH-independent second-order rate constant for reaction with one inhibitor and $\tilde{k}_2$ is the analogous rate constant in the same ionization state for reaction with another inhibitor so that, when the structural change provides that $\tilde{k}_2 > \tilde{k}_1$, $\Delta\Delta G_{\text{KS}}$ is positive). 3. The kinetic data further illuminate the nature of the interdependence of binding interactions in papain first noted by Kowlessur, Topham, Thomas, O'Driscoll, Templeton & Brocklehurst [(1989) *Biochem. J.* **258**, 755–764] in the S₂ subsite, S₁–S₂ intersubsite and catalytic-site regions. Of particular note is the apparent dependence of the binding of the *N*-Ac-D-Phe moiety on the binding of the leaving group to (His-159)–Im⁺–H and the fact that the resulting rate enhancement is more effective when (P₁)–N–H is absent than when it is present. This result revealed by kinetic analysis goes beyond the conclusion suggested by model building that it is possible to make all of the binding contacts in complexes involving the D-enantiomers [(II) and (IV)] as in those involving the L-enantiomers [(I) and (III)]. 4. Removal of the opportunity for formation of the (P₁)–N–H...O=C < (Asp-158) hydrogen bond results in a substantial decrease in the index of stereochemical selectivity [$I_{\text{ss}} = \tilde{k}_{\text{L}}/\tilde{k}_{\text{D}}$ for inhibitors and corresponds to $(\tilde{k}_{\text{cat}}/\tilde{k}_{\text{m}})_{\text{L}}/(\tilde{k}_{\text{cat}}/\tilde{k}_{\text{m}})_{\text{D}}$ for substrates]. The perceived binding modes of the two pairs of enantiomeric inhibitors and of the analogous enantiomeric substrates (Ac-Phe-Gly 4-nitroanilides) are discussed in relation to the values of I_{ss} .

INTRODUCTION

The study of molecular recognition [see Roberts (1989) for reviews] involves the assignment of roles to various types of non-covalent interaction (electrostatic, hydrogen-bonding, van der Waals and hydrophobic) to account for the selective binding of one molecule with another. To understand molecular recognition in enzyme–substrate and enzyme–time-dependent inhibitor systems, however, it is necessary to consider not only binding *per se*, but also the interdependence of binding interactions and the covalency changes that have a central role in catalytic-site chemistry. This dynamic aspect of molecular recognition is one of the least well understood aspects of enzyme chemistry, and one of the most sensitive and direct ways of investigating catalytic-site geometry and its modulation by ligand binding is the study of chemical-reactivity characteristics. Transition-state geometry is the fundamental characteristic on which catalytic ability depends, and the reactivity of a catalytic-site nucleophile that plays a central role in the catalytic act is a prime target for investigation of factors that contribute to the control of transition-state geometry. The cysteine proteinase papain (EC 3.4.22.2) provides valuable opportunities for the study of the interdependence of binding interactions with each other and with

catalytic-site chemistry (Kowlessur *et al.*, 1989b). The reactivity of its catalytic site responds to combinations of hydrogen-bonding and hydrophobic effects in its extended binding site (Brocklehurst *et al.*, 1987a, 1988a,b; Kowlessur *et al.*, 1989a,b, 1990; Templeton *et al.*, 1990; Berti *et al.*, 1991; Hanzlik *et al.*, 1991; Patel *et al.*, 1992). Papain is the best-characterized member of the cysteine proteinase family both structurally and mechanistically [see Brocklehurst (1987) and Brocklehurst *et al.* (1987b) for reviews, and Varughese *et al.* (1989), Bjork & Ylinenjarvi (1990), Stubbs *et al.* (1990), Menard *et al.* (1990, 1991a–c), Khouri *et al.* (1991), Vernet *et al.* (1991), Pickersgill *et al.* (1992), Harris *et al.* (1992) and Lindahl *et al.* (1992) for recent crystallographic, spectroscopic and molecular biological studies that further illuminate structure, ligand binding and catalytic mechanism].

In the present paper we report the synthesis and characterization of 2-(*N*'-acetyl-D-phenylalanyl)hydroxyethyl 2'-pyridyl disulphide [compound (IV)] and the results of a study of the pH-dependence of the second-order rate constant (*k*) for its reaction with the thiol group of Cys-25 of papain. These new data were required to complement existing pH-dependent kinetic data including those for the reactions of papain with compound (III) [the L-enantiomer of compound (IV)] (Kowlessur *et al.*,

‡ To whom correspondence should be sent.

1989b) and the L- and D-enantiomers of 2-(*N'*-acetylphenylalanyl)aminoethyl 2'-pyridyl disulphide [compounds (I) and (II) respectively] (Brocklehurst *et al.*, 1988b; Templeton *et al.*, 1990) to permit an assessment of the contributions of the (P_1)-N-H...O=C < (Asp-158) and (P_2) > C=O...H-N-(Gly-66) hydrogen bonds and enantiomeric P_2 -S₂ hydrophobic contacts in papain-ligand association in two manifestations of dynamic molecular recognition: (i) signalling to the catalytic-site region to provide for an Im⁺-H-assisted transition state and (ii) the dependence of P_2 -S₂ stereochemical selectivity on hydrogen-bonding interactions outside the S₂ subsite.

MATERIALS AND METHODS

Materials

Papain. Papain was the 2× crystallized product supplied by Sigma (Poole, Dorset, U.K.) as a suspension in 0.05 M-sodium acetate buffer, pH 4.5. In preparation for a set of kinetic experiments, 0.5 ml of the suspension was mixed with an equal volume of 40 mM-cysteine solution in sodium pyrophosphate buffer, pH 8.0, *I* 0.3 and allowed to stand for 30 min at room temperature (approx. 22 °C) to convert any reversibly oxidized papain into active enzyme. Low-*M_r* material was then removed by gel filtration on a Sephadex G-25 column (15.0 cm × 2.5 cm). Elution with 0.1 M-KCl containing 1 mM-EDTA and collection of approx. 10 ml fractions produced activator-free papain in approx. 10 ml of eluate after approx. 30 ml had been collected and discarded; 10–12 ml of the post-30 ml eluate contains sufficient papain for approx. 30–40 stopped-flow kinetic runs. Papain thus prepared was shown to be free of contaminant chymopapains both by thiol titration with 2,2'-dipyridyl disulphide (2-Py-S-S-2-Py) at pH 4 and 8 (Baines & Brocklehurst, 1978) and by f.p.l.c. analysis with Pharmacia LKB equipment on a Mono S HR5/5 column. Fully active papain containing 1 mol of thiol and 1 mol of intact catalytic-site Cys-25/His-159 interactive system is conveniently prepared as required by covalent chromatography (Brocklehurst *et al.*, 1985). A molecular model of papain based on the co-ordinates of Drenth *et al.* (1976) was supplied by Labquip, Reading, Berks, U.K.

2-(*N'*-Acetyl-D-phenylalanyl)hydroxyethyl 2'-pyridyl disulphide [compound (IV)]. 2-Hydroxyethyl 2'-pyridyl disulphide hydrochloride (Brocklehurst *et al.* 1988b) (223 mg, 1 mmol) was suspended in *NN'*-dimethylformamide (5 ml) at 4 °C. Triethylamine (140 µl, 1 mmol) was added and the mixture was allowed to stir for 30 min. The following additions were then made sequentially with stirring: *N*-*t*-butyloxycarbonyl-D-phenylalanine (265 mg, 1 mmol), dicyclohexylcarbodi-imide (206 mg, 1 mmol) and 4-(*NN'*-dimethylamino)pyridine (10 mg). The mixture was allowed to stir for 5 h at 4 °C and then overnight at room temperature before being added with stirring to an excess of an ice/water mixture (approx. 50 ml). After the ice had melted, the quenched reaction mixture was extracted with methylene dichloride (2 × 25 ml). The combined methylene dichloride extracts were dried over anhydrous Na₂SO₄ and, after filtration, the solvent was removed by rotary evaporation *in vacuo*. The residue was dissolved in methylene dichloride (5 ml) at 15 °C and trifluoroacetic acid (5 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. NaHCO₃ and ethyl acetate, and the ethyl acetate layer was dried over anhydrous Na₂SO₄. After removal of the drying agent by filtration, the filtrate was treated sequentially with triethylamine (0.28 ml, 2 mmol) and acetic anhydride (0.2 ml, 2 mmol), and the mixture was stirred at room temperature for 15 min. 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 homogeneous product, which crystallized slowly over 1–2 weeks on storage at 4 °C. It was recrystallized from cold ethyl acetate to which hexane was added to turbidity, and then melted sharply. It had m.p. 59 °C and $[\alpha]_D^{20} = -6.6 \pm 0.1^\circ$ (*c* 1.2 in methanol). A sample produced the predicted yield of pyridine-2-thione consequent on 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). The i.r. spectrum was identical with that of the L-enantiomer [compound (III); Kowlessur *et al.*, 1989a]. Differences in the synthetic routes used to prepare the L- and D-enantiomers compounds (III) and (IV) and the characteristics of the two enantiomers are briefly discussed in the Results and discussion section.

Kinetics

All reactions were studied at 25 °C and *I* 0.1 in solutions containing 1 mM-EDTA by using an Applied Photophysics SF.17MV stopped-flow spectrophotometer, kinetics workstation and data-acquisition and -analysis software. Release of pyridine-2-thione consequent on reaction of the papain thiol group with compound (IV) was monitored at 343 nm. The buffers were as described by Brocklehurst *et al.* (1987a). All reactions were carried out under pseudo-first-order conditions with [papain-SH] = 2–3 µM and [compound (IV)] = 30–300 µM. The (pseudo)-first-order rate constant ($\tilde{k}_{obs}$) increased linearly with increase in [compound (IV)] at pH 6.0, thus establishing adherence to overall second-order kinetics under the conditions of concentration used.

Evaluation of pH-dependent kinetic data

Kinetic models differing in the number and characteristics of reactive hydronic states were evaluated by using a multitasking application program (SKETCHER) written in ANSI C (Kernighan & Ritchie, 1988) running under RISCOS on an Acorn Archimedes microcomputer (Brocklehurst *et al.*, 1990; Topham *et al.* 1991). Rate equations for reactions in a variety of hydronic states were written down by using the simple general expression and two information matrices described by Brocklehurst *et al.* (1990) and Topham *et al.* (1991). The eight-parameter (four-pK_a) rate equation that best describes the pH-dependence of the reaction of papain with compound (IV) is eqn. (1) (see Fig. 1 of the Results and discussion section). The pH-*k* data and theoretical lines were displayed by using a Compaq Deskpro 386/20e PC and Hewlett-Packard Colour Pro Plotter. Difference kinetic specificity energies, $\Delta\Delta G_{KS}$ (see the Results and discussion section), were calculated for *T* = 298K by using $\Delta\Delta G_{KS} = -8.31 \times 298 \ln(\tilde{k}_1/\tilde{k}_2) \times 10^{-3} = -2.476 \ln(\tilde{k}_1/\tilde{k}_2)$ kJ·mol⁻¹.

RESULTS AND DISCUSSION

Synthesis and characteristics of the enantiomeric reactivity probes [compounds (III) and (IV)]

The synthesis of the L-enantiomer [compound (III)] reported previously (Kowlessur *et al.*, 1989b) included the acylation of 2-hydroxyethyl 2'-pyridyl disulphide free base with *N*-*t*-butyloxycarbonyl-L-phenylalanine *N'*-hydroxysuccinimide ester. This is a slow reaction and it was necessary to keep the reactants for 48 h at room temperature in the presence of approx. 0.1 mM-4-(*NN'*-dimethylamino)pyridine, a situation in which racemization might occur. In the present work, the synthesis of the D-enantiomer [compound (IV)] was achieved under less harsh conditions [25 µM-4-(*NN'*-dimethylamino)pyridine; 5 h at 4 °C and overnight at room temperature] in a one-pot synthesis from 2-hydroxyethyl 2'-pyridyl disulphide hydrochloride to 2-(*N'*-*t*-

Fig. 1. pH-dependence of k for the reaction of papain with 2-(*N'*-acetyl-D-phenylalanyl)hydroxyethyl 2'-pyridyl disulphide (IV) at 25 °C and /0.1 in aqueous buffers

The points are experimental and the continuous line is theoretical for eqn. (1), with the following values of the characterizing parameters with values of $\bar{k}$ in $\text{M}^{-1} \cdot \text{s}^{-1}$:

$$\bar{k}_{\text{XH}_3} = 1.3 \times 10^5, \bar{k}_{\text{XH}_2} = 1.45 \times 10^5, \bar{k}_{\text{XH}} = 3.5 \times 10^4, \bar{k}_{\text{X}} = 2.1 \times 10^4, \text{p}K_{\text{XH}_4} = 3.7, \text{p}K_{\text{XH}_3} = 3.8, \text{p}K_{\text{XH}_2} = 8.1, \text{p}K_{\text{XH}} = 9.5$$

$$k = \frac{\bar{k}_{\text{XH}_3}}{1 + \frac{[\text{H}^+]}{K_{\text{NH}_1}} + \frac{K_{\text{XH}_4}}{[\text{H}^+]} + \frac{K_{\text{XH}_3}K_{\text{XH}_2}}{[\text{H}^+]^2} + \frac{K_{\text{XH}_3}K_{\text{XH}_2}K_{\text{XH}}}{[\text{H}^+]^3}} + \frac{\bar{k}_{\text{XH}_2}}{1 + \frac{[\text{H}^+]^2}{K_{\text{XH}_4}K_{\text{XH}_3}} + \frac{[\text{H}^+]}{K_{\text{XH}_3}} + \frac{K_{\text{XH}_2}}{[\text{H}^+]} + \frac{K_{\text{XH}_2}K_{\text{XH}}}{[\text{H}^+]^2}} + \frac{\bar{k}_{\text{XH}}}{1 + \frac{[\text{H}^+]^3}{K_{\text{XH}_4}K_{\text{XH}_3}K_{\text{XH}_2}} + \frac{[\text{H}^+]^2}{K_{\text{XH}_3}K_{\text{XH}_2}} + \frac{[\text{H}^+]}{K_{\text{XH}_2}} + \frac{K_{\text{XH}}}{[\text{H}^+]}} + \frac{\bar{k}_{\text{X}}}{1 + \frac{[\text{H}^+]^4}{K_{\text{XH}_4}K_{\text{XH}_3}K_{\text{XH}_2}K_{\text{XH}}} + \frac{[\text{H}^+]^3}{K_{\text{XH}_3}K_{\text{XH}_2}K_{\text{XH}}} + \frac{[\text{H}^+]^2}{K_{\text{XH}_2}K_{\text{XH}}} + \frac{[\text{H}^+]}{K_{\text{XH}}}} \quad (1)$$

The dotted lines correspond to contributions to k of the individual hydronic states of the reaction which are provided by the terms in eqn. (1) associated with $\bar{k}_{\text{XH}_3}$ – $\bar{k}_{\text{X}}$ respectively.

butyloxycarbonyl-D-phenylalanyl)hydroxyethyl 2'-pyridyl disulphide by using dicyclohexyldicarbodi-imide as coupling agent. The somewhat larger numerical (negative) value of $[\alpha]_D^{20}$ obtained for the D-enantiomer ($-6.6 \pm 0.1^\circ$) than for the L-enantiomer ($+5.9 \pm 0.1^\circ$) suggests that some racemization might have occurred in the synthesis of the L-enantiomer. There is no evidence, however, for the presence of discernible D-enantiomer from the single-phase progress curves for complete reaction of papain with compound (III) under equimolar conditions (Kowlessur *et al.*, 1989a). The finding in the present work that the reaction of papain with the L-enantiomer (III) is almost 10 times faster than that of its reaction with the D-enantiomer (IV) supports the view that contamination of (III) with (IV) and vice versa is negligible and does not distort the values of the first-order rate constants determined for the stoichiometric reactions with papain.

pH-dependent kinetics of the reactions of papain with 2-(*N'*-acetyl-D-phenylalanyl)hydroxyethyl 2'-pyridyl disulphide [compound (IV)] and with the related disulphides [compounds (I)–(III), (V) and (VI)]

The main features of the shape of the pH- k profile for this reaction (Fig. 1) are similar to those of the analogous profiles for the reactions of papain with the L-enantiomer [compound (III)] (Kowlessur *et al.*, 1989b) and the enantiomeric 2-(*N'*-acetyl-phenylalanyl)amino)ethyl 2'-pyridyl disulphides [compounds (I) and (II)] (Brocklehurst *et al.*, 1988b; Templeton *et al.*, 1990) (Figs. 2b and 2c). The shapes of these profiles contrast markedly with the shape of the corresponding profile for the reaction of papain with 2-(acetoxyl)-ethyl 2'-pyridyl disulphide [compound (VI)] which lacks a phenylalanine residue at P₂ and contains a P₁–P₂ ester bond instead of a P₁–P₂ amide bond (Brocklehurst *et*

al., 1988b) [Fig. 2(a), curve (ii)]. This profile resembles those for the reactions of papain with featureless probes containing only simple hydrocarbon side chains (such as $\text{CH}_3\text{--CH}_2\text{--CH}_2\text{--S--S--2-Py}$) (Brocklehurst *et al.*, 1988b) which are characterized by a rate minimum at pH 6–7 instead of a rate maximum. The shape of the profile for the reaction with 2-(acetamido)ethyl 2'-pyridyl disulphide [compound (VII)], which contains a P₁–P₂ amide bond but which lacks an L-Phe residue at P₂ (Brocklehurst *et al.*, 1988b) [Fig. 2(a), curve (i)], is intermediate between the shape of that in Fig. 2(a) [curve (ii)] and those in Fig. 1 and Figs. 2(b) and 2(c).

- (I) X = NH, L-enantiomer
- (II) X = NH, D-enantiomer
- (III) X = O, L-enantiomer
- (IV) X = O, D-enantiomer

- (V) X = NH
- (VI) X = O

Fig. 2. Variation in the shape of the pH- k profile for reactions of papain with time-dependent inhibitors (R-S-S-2-Py) with change in inhibitor structure

The continuous lines correspond to eqn.(1) of Fig. 1 legend and the values of the characterizing parameters taken from Brocklehurst *et al.* (1988b), Kowlessur *et al.* (1989a), Templeton *et al.* (1990) and Fig. 1, and collected in Table 1: for (a) curve (i) R = Ac-NH-(CH₂)₂ (compound V), curve (ii) R = Ac-O-(CH₂)₂ (compound VI); for (b) curve (i) R = N-Ac-(L-Phe)-NH-(CH₂)₂, curve (ii) R = N-Ac-(L-Phe)-O-(CH₂)₂; for (c) curve (i) R = N-Ac-(D-Phe)-NH-(CH₂)₂, curve (ii) R = N-Ac-(D-Phe)-O-(CH₂)₂. The arrows define the limits of the data.

Potential binding interactions between papain and the substrate-related inhibitors

The thiol-specific inhibitors (I)–(VI) were designed to provide a chromophoric product (pyridine-2-thione) resulting from nucleophilic attack by the thiolate anion component of the Cys-25/His-159 ion-pair system of the papain catalytic site with the

possibility of assistance to leaving-group departure by association with (His-159)–Im⁺H (Scheme 1c) and opportunities to engage in some or all of the following binding interactions in the S₂ subsite and S₁–S₂ intersubsite regions: (P₂)–NH...O=C < (Gly-66), (P₂) > C=O...H–N–(Gly-66), (P₂)–CH₂Ph(S₂) and (P₁)–NH...O=C < (Asp-158) (Scheme 1a). The hydrogen-bonding and P₂–S₂ hydrophobic contacts are the main molecular-recognition determinants for papain (see Drenth *et al.*, 1976; Asboth *et al.*, 1988; Templeton *et al.*, 1990; Kowlessur *et al.*, 1990; Berti *et al.*, 1991).

Analysis and interdependence of binding interactions with each other and with catalytic-site chemistry

Systematic change in the structure of the inhibitor permits kinetic assessment of the effects of individual interactions such as specific hydrogen bonds on the behaviour of the enzyme, and the resulting changes in rate constants may be expressed as difference kinetic specificity energies, $\Delta\Delta G_{KS}$, defined by eqn. (2):

$$\Delta\Delta G_{KS} = -RT \ln(\tilde{k}_1/\tilde{k}_2) \quad (2)$$

where $\tilde{k}_1$ is the pH-independent second-order rate constant for reaction with one inhibitor and $\tilde{k}_2$ is the pH-independent second-order rate constant for reaction in the same ionization state with another inhibitor. If the structural change is such that $\tilde{k}_2 > \tilde{k}_1$, $\Delta\Delta G_{KS}$ is positive. $\Delta\Delta G_{KS}$ is analogous to the apparent binding energy or incremental specificity constant, given the symbol ΔG_{app} , by Fersht (1988) and used in studies on structural variants of enzymes, and $\Delta\Delta G_{obs}$, by Berti *et al.* (1991) and adapted for use with experiments on structural variants of substrates. These difference free-energy changes provide lower limits of the values of the incremental binding energies (Fersht, 1988).

The interdependence of binding interactions in papain, first noted by Kowlessur *et al.* (1989b) and investigated by Berti *et al.* (1991) using steady-state substrate kinetics, is discussed below in connection with the substrate-related 2-pyridyl disulphide inhibitors. The dependence of catalytic-site chemistry on particular combinations of binding interactions may be revealed by comparison of reactivities in particular ionization states (Kowlessur *et al.*, 1989b). Thus relative reactivities through the transition

Scheme 1. Major binding interactions and transition states postulated for reactions of papain with alkyl 2-pyridyl disulphide reactivity probes

(a) Binding in the S₁–S₂ intersubsite and S₂ subsite regions of papain is considered to involve the three hydrogen bonds involving the backbone carbonyl group of Asp-158 and the backbone carbonyl and amino groups of Gly-66, and the hydrophobic S₂ subsite in which the benzyl side chain of the phenylalanine residue is accommodated; details of these interactions and of others in the catalytic-site region for both L- and D-enantiomers are shown in Scheme 2; (b) transition state for the unassisted reaction of the uncomplicated thiolate anion of Cys-25 in the X-state at high pH; (c) transition state for the reaction of the thiolate anion component of the catalytic-site Cys-25/His-159 ion pair assisted by association of the imidazolium cation component with the N atom of the 2-mercaptopyridine leaving group.

state shown in Scheme 1(b) (X-state reactivities) probably reflect relative binding abilities of the inhibitor. By contrast, reactivities in ionization states capable of providing for reaction through the transition state shown in Scheme 1(c) (XH or XH₂ state reactivities) reveal binding-site-catalytic-site signalling effects resulting from Im⁺-H-assisted reactions. These appear as deviations from the analogous reactivity ratios in the X-state and some structural changes can produce rate maxima instead of rate minima.

Effects of binding interactions on catalytic-site reactivities in individual ionization states

Reactivities of the uncomplicated thiolate anion of Cys-25 towards the non-hydrated inhibitors (the X-state). These reactivities (the values of $\tilde{k}_x$ in Table 1) are those in the plateau regions at high pH (pH greater than 10) of Figs. 1 and 2 and relate to anionic transition states of the type shown in Scheme 1(b). For the reaction of papain with CH₃-CH₂-CH₂-S-S-2-Py, the value of $\tilde{k}_x$ is $1.8 \times 10^3 \text{ M}^{-1} \cdot \text{s}$ (Brocklehurst *et al.* 1988a). For its reactions with compounds (II) and (IV)-(VI), the values of $\tilde{k}_x$ are all approx. $2 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Table 1). Thus introduction of the opportunity for formation of a (P₂) > C=O...H-N-(Gly-66) hydrogen bond [compound (VI)] produces an increase in $\tilde{k}_x$ of about an order of magnitude from its value for reaction of the inhibitor with a featureless (*n*-propyl) side chain. This corresponds to a $\Delta\Delta G_{\text{KS}}$ value of $+5.68 \text{ kJ} \cdot \text{mol}^{-1}$. It is noteworthy that this rate enhancement is not further changed substantially by provision additionally of the opportunity for formation of the (P₁)-NH...O=C < (Asp-158) hydrogen bond either alone [compound (V)] or in combination with the possibility of hydrophobic contacts with the D-Phe side chain in the S₂ subsite [compound (II)]. The same lack of additional rate enhancement applies when the opportunity for hydrophobic contact in the absence of the (P₁)-NH...O=C < (Asp-158) hydrogen bond is provided in the D-enantiomer [compound (IV)]. By contrast, when the opportunity for hydrophobic contact with the L-enantiomer is added to that for (P₂) > C=O...H-N-(Gly-66) hydrogen-bond formation, i.e. (VI) → (III), there is an additional 7-fold increase in $\tilde{k}_x$ ($\Delta\Delta G_{\text{KS}} = +5.0 \text{ kJ} \cdot \text{mol}^{-1}$) and when the opportunity for (P₁)-NH...O=C < (Asp-158) hydrogen-bond formation is added to these two contacts, i.e. (III) → (I), there is an additional 5-fold increase in $\tilde{k}_x$ ($\Delta\Delta G_{\text{KS}} = 4.2 \text{ kJ} \cdot \text{mol}^{-1}$). Thus, whereas addition of opportunity for both the D-type of hydrophobic contact and the (P₁)-NH...O=C < (Asp-158) hydrogen bond to that for the (P₂) > C=O...H-N-(Gly-66) hydrogen bond, i.e. (VI) → (II), produces no increase in $\tilde{k}_x$, addition of both the L-type hydrophobic contact and the (P₁)-NH...O=C < (Asp-158) hydrogen bond, i.e. (VI) → (I), produces a 36-fold increase in $\tilde{k}_x$ ($\Delta\Delta G_{\text{KS}} = 8.8 \text{ kJ} \cdot \text{mol}^{-1}$). The conclusion therefore is that for the reactions in the X-state the (P₂) > C=O...H-N-(Gly-66) hydrogen bond provides a positive binding interaction which is co-operative with P₂-S₂ hydrophobic contacts in the L-enantiomer-binding mode and with the (P₁)-NH...O=C < (Asp-158) hydrogen bond, but only if the L-P₂-S₂ contacts exist. P₂-S₂ hydrophobic contacts in the D-enantiomer binding mode do not provide discernible additional binding in either the presence or absence of the (P₁)-NH...O=C < (Asp-158) hydrogen bond and additional binding is not provided either by this hydrogen bond in the absence of L-P₂-S₂ contacts.

Reactivities in ionization states in which the contribution of the (His-159)-Im⁺H-assisted reaction of the thiolate anion of Cys-25 with the non-hydrated inhibitors is maximal (XH or XH₂ states). The ionization states in question are those in which *k* is a minimum of Fig. 2(a), curve (ii) and in the pH-*k* profile for the reaction of papain with CH₃-CH₂-CH₂-S-S-2-Py (Brocklehurst *et al.*, 1988a) (both at pH values approx. 7) and a maximum in

Table 1. 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; the shapes of the pH-*k* profiles are shown in Figs. 1 and 2.

Probe	Chemical structure	Macroscopic pK _a values			pH-independent rate constants (M ⁻¹ ·s ⁻¹)			
		pK _{XH₄}	pK _{XH₃}	pK _{XH₂}	10 ⁻⁴ × $\tilde{k}_{\text{XH}_4}$	10 ⁻⁴ × $\tilde{k}_{\text{XH}_3}$	10 ⁻⁴ × $\tilde{k}_{\text{XH}_2}$	10 ⁻⁴ × $\tilde{k}_x$
(I)	CH ₃ -C(=O)-NH-CH(CH ₂ Ph)-C(=O)-NH-[CH ₂] ₂ -S-S-2-Py	3.9	4.1	8.25	300	610	230	60
(II)	L-isomer	3.7	4.3	7.9	10	20	4	1.5
(III)	D-isomer							
(IV)	CH ₃ -C(=O)-NH-CH(CH ₂ Ph)-C(=O)-O-[CH ₂] ₂ -S-S-2-Py	3.9	4.2	8.2	90	120	60	11.5
(V)	L-isomer	3.7	3.8	8.1	13	14.5	3.5	2.1
(VI)	D-isomer							
(VII)	CH ₃ -C(=O)-NH-[CH ₂] ₂ -S-S-2-Py	2.48*	3.7	3.7	0.1	2.95	2.95	1.95
(VIII)	L-isomer							
(IX)	D-isomer							
(X)	CH ₃ -C(=O)-O-[CH ₂] ₂ -S-S-2-Py	3.75	3.75	5.0*	3.5	1.0	0.58	1.65

* pK_a 2.48 characterizes hydronic dissociation from the conjugate acid of probe (V) from the pyridyl nitrogen atom; pK_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 pK_a values of approx. 4 and two in alkaline media with pK_a values of approx. 8.2 and 9.5, but for the reaction with probe (V), the pK_a of 9.5 cannot be discerned, possibly because of the small amplitude of the sigmoid wave linking the XH and X states.

Table 2. Effects of various combinations of the following postulated molecular-recognition features on the relative reactivities of individual hydronic states ($X\text{-XH}_2$) and on the index of stereochemical selectivity (I_{ss}) for reactions of papain with substrate-derived 2-pyridyl disulphides $R\text{-S-S-2-Py}$: (a) interaction with the L-phenylalanine side chain in the S_2 subsite, (b) hydrogen-bonding of the backbone N-H of Gly-66 to the (P_2)-carbonyl oxygen atom and (c) hydrogen-bonding of the (P_1)-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 dissociation (see Table 1).

Probe	Side chain	Postulated binding interaction(s)	$\frac{\tilde{k}_{XH}}{\tilde{k}_X}$	$\frac{\tilde{k}_{XH_2}}{\tilde{k}_X}$	Index of stereochemical selectivity			
					$I_{ss} = (\tilde{k}_{XH_n})_L/(\tilde{k}_{XH_n})_D$			
					$n = 0$	$n = 1$	$n = 2$	$n = 3$
(I)	$\begin{array}{c} \text{O} \\ \\ \text{CH}_3\text{-C-NH-CH(CH}_2\text{Ph)-C-NH-[CH}_2\text{]}_2\text{-} \\ \text{L-isomer} \end{array}$	(a), (b) and (c)	3.8	10.2*	40	57.5	30.5	30
(II)	$\begin{array}{c} \text{O} \\ \\ \text{CH}_3\text{-C-NH-CH(CH}_2\text{Ph)-C-NH-[CH}_2\text{]}_2\text{-} \\ \text{D-isomer} \end{array}$		2.7	13.3*				
(III)	$\begin{array}{c} \text{O} \qquad \qquad \qquad \text{O} \\ \qquad \qquad \qquad \\ \text{CH}_3\text{-C-NH-CH(CH}_2\text{Ph)-C-O-[CH}_2\text{]}_2\text{-} \\ \text{L-isomer} \end{array}$	(a) and (b)	5.2	10.4*	5.5	17	8.3	6.9
(IV)	$\begin{array}{c} \text{O} \qquad \qquad \qquad \text{O} \\ \qquad \qquad \qquad \\ \text{CH}_3\text{-C-NH-CH(CH}_2\text{Ph)-C-O-[CH}_2\text{]}_2\text{-} \\ \text{D-isomer} \end{array}$		1.7	6.9*				
(V)	$\begin{array}{c} \text{O} \\ \\ \text{CH}_3\text{-C-NH-[CH}_2\text{]}_2\text{-} \end{array}$	(b) and (c)	1.5*	(1.5)				
(VI)	$\begin{array}{c} \text{O} \\ \\ \text{CH}_3\text{-C-O-[CH}_2\text{]}_2\text{-} \end{array}$		0.3*	(0.6)				

* These six values provide a comparison of the relative reactivities in the pH region 6–7, where k is a maximum [$\tilde{k}_{XH}$ for (I)–(IV) and $\tilde{k}_{XH}$ for probe (V) or a minimum $\tilde{k}_{XH}$ for probe (VI)] and in the plateau region at high pH; the values of $\tilde{k}_{XH}$ for probes (I)–(IV) 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 1).

Figs. 1, 2(a) [curve (i)], 2(b) and 2(c) (all at pH values approx. 6). Where k is a maximum at pH approx. 6, reaction is considered to proceed to a significant extent through a (His-159)-Im⁺H-assisted transition state of the type shown in Scheme 1(c). The ionization states to be compared are the XH states for the reactions of compounds (V), (VI) (Table 1) and of $\text{CH}_3\text{-CH}_2\text{-CH}_2\text{-S-S-2-Py}$ (Brocklehurst *et al.*, 1988a) and the XH_2 states for the reactions of (I)–(IV) (Table 1). In the case of the reactions of (I)–(IV), there are additional (XH) states of intermediate reactivity where reaction via the transition state of Scheme 1(c) is not optimal [for an example, see Fig. 1 where contributions from reactions in individual ionization states corresponding to individual terms in eqn. (1) are displayed]. Perturbation of the reactions of the Cys-25/His-159 ion pair of papain by pH-dependent phenomena leading to these intermediate states has been discussed by Brocklehurst *et al.* (1988b) and is not considered further in the present paper.

Provision of the opportunity for the hydrogen-bonding interaction between (His-159)-Im⁺H and the leaving group of the inhibitor results in consequences of the stepwise addition of other binding opportunities that are different in some cases from those discussed above that occur when the hydrogen bond to the leaving group does not exist [the X-state reactivities (see Table 3 below)]. For example, the change from an *n*-propyl side chain in the inhibitor to that in (VI) which provides for the (P_2) > C=O...H-N-(Gly-66) hydrogen bond results in a similar fold increase in $\tilde{k}_{XH}$ (7-fold from $8.0 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ to $5.8 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$; $\Delta\Delta G_{KS} = 5.0 \text{ kJ} \cdot \text{mol}^{-1}$) as in $\tilde{k}_X$ (9-fold from 1.8×10^3 to $1.65 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$; $\Delta\Delta G_{KS} = 5.4 \text{ kJ} \cdot \text{mol}^{-1}$) and does not produce the rate maximum at pH 6 [Fig. 2a (curve ii)] (indicative of a major contribution to reaction via the transition state shown in Scheme 2c). By contrast, provision additionally of opportunity for formation of a (P_1)-N-H...O=C < (Asp-158) hydrogen bond [(VI) → (V)] results in a further 5-fold increase in

$\tilde{k}_{XH}$ ($\Delta\Delta G_{KS} = 4.2 \text{ kJ} \cdot \text{mol}^{-1}$). Similarly, augmentation of the (P_2) > C=O and (P_1)-N-H potential binding sites by addition of the *N*-Ac-D-Phe moiety [(V) → (II)] provides no rate enhancement to the reaction in the X-state but a further 7-fold enhancement in the XH_2 state when the hydrogen bond to the leaving group exists. Addition instead of the *N*-Ac-L-Phe moiety [(V) → (I)] provides a further 30-fold enhancement in the X state and a 207-fold enhancement in the XH_2 state. Addition of the *N*-Ac-L-Phe moiety to the inhibitor containing (P_2) > C=O produces the same (207-fold) rate enhancement ($\Delta\Delta G_{KS} = 13.4 \text{ kJ} \cdot \text{mol}^{-1}$) in the XH_2 state whether the (P_1)-N-H hydrogen-bond donor is present or whether it is replaced by (P_1)-O. When the hydrogen to the leaving group is not also present (X-state reactivities), however, the rate enhancement is less (7-fold) in the absence of (P_1)-N-H [(VI) → (II)] than when this feature is present [30-fold; (V) → (I)]. Addition of the *N*-Ac-D-Phe moiety to (P_2) > C=O in the absence of (P_1)-N-H [(VI) → (IV)] provides a larger rate enhancement (25-fold) in the XH_2 state than when (P_1)-N-H is present [7-fold; (V) → (II)]. When the hydrogen bond to the leaving group is not present, however, addition of the *N*-Ac-D-Phe moiety is essentially without net effect whether (P_1)-N-H is present [(V) → (II)] or not [(VI) → (IV)]. Addition of (P_1)-NH to the inhibitor containing both (P_2) > C=O and *N*-Ac-D-Phe [(IV) → (II)] results in little change in reactivity whether or not the hydrogen bond to the leaving group exists. Addition of the same feature to the inhibitor containing (P_2) > C=O and *N*-Ac-L-Phe produces a 5-fold increase in reactivity but this is not dependent on the presence of the hydrogen bond to the leaving group.

The results of the above analysis, summarized in Table 3, demonstrate the interdependence of binding interactions in the S_2 subsite, in the S_1 - S_2 intersubsite region, and in the catalytic site of papain. One phenomenon that has been revealed is the dependence of the kinetically effective binding of the *N*-Ac-D-Phe moiety on the binding of the leaving group to (His-159)-Im⁺H.

Scheme 2. Diagram showing postulated binding modes for the transition states in the reaction of the thiol group of papain with (a) *N*-acetyl-L-Phe-NH-(CH₂)₂-S-S-2-Py, (b) its D-enantiomer, (c) *N*-acetyl-L-Phe-O-(CH₂)₂-S-S-2-Py and (d) its D-enantiomer

Each drawing shows a view looking into the active-centre cleft of the enzyme molecule with the L and R domains towards the top and bottom respectively of the field. Covalent bond lengths are approximately to scale but enzyme-ligand interatomic distances have been exaggerated for clarity. The placing of enzyme side chains and backbone groups is not topographically correct, but shows their relative dispositions around the cleft and their approximate spatial relationships to the ligand. The small curly arrows indicate the changes in electron density required for the expulsion of the 2-thiopyridone leaving group consequent on nucleophilic attack by the thiolate anion of Cys-25 and assisted by association of the N atom of the pyridyl ring with (His-159)-Im⁺H. The large ribbon arrows indicate the sense in which the D-enantiomeric ligand moieties need to be rotated about their long axes, from their perceived positions in the adsorptive complexes, in order to obtain improved binding of the phenylalanine side chain in the S₂ subsite.

In the absence of this hydrogen bond, the *N*-Ac-D-Phe moiety does not appear to bind significantly to papain. When the hydrogen bond to the leaving group exists, however, the presence of the *N*-Ac-D-Phe moiety provides significant rate enhancement and this is more effective when (P₁)-N-H is absent than when it is present.

Stereochemical selectivity

The net effects of the interactions discussed above on stereochemical selectivity are recorded in Table 2, as values of *I*_{ss} (index of stereochemical selectivity). The most obvious result is that removal of the opportunity for hydrogen-bonding of (P₁)-NH to the backbone carbonyl oxygen of Asp-158 results in a substantial decrease in the value of *I*_{ss}. Neglecting the values of *I*_{ss} for the XH states (where the estimates of *k* will be most prone to uncertainty, see Fig. 1), the effect of replacing (P₁)-NH by (P₁)-O is a decrease in *I*_{ss} from 30–40 to approx. 7. Model building demonstrates the possibility of making the required binding contacts in the papain active centre for both enantiomeric pairs of inhibitors (Scheme 2a–d). The active-centre cleft is readily bridged by the (P₁-P₂) amide bond of compounds (I) and (H), and a satisfactory P₂-S₂ interaction is possible in both cases

(Schemes 2a and 2c). For both of these enantiomers, the aromatic ring of (P₂)-Phe appears to be able to occupy a very similar position in the active centre, sandwiched between the side chains of Val-133 and Val-157. The position of the methylene group of (P₂)-Phe differs. For the L-enantiomer it lies near to Pro-68 and Ala-160 (Scheme 2a) whereas it projects into solvent for the D-enantiomer (Scheme 2c) with consequent predicted loss of binding energy. With each enantiomer, near-optimal alignment of the reacting centres in the inhibitor with the catalytic-site groups of the enzyme is possible without perturbing the other enzyme-ligand interactions. This contrasts with the situation for the interaction of papain with enantiomeric Ac-Phe-Gly 4-nitro-anilides (Kowlessur *et al.*, 1990; Patel *et al.*, 1992), where rotational adjustments are necessary and the *I*_{ss} value is considerably greater. The difference between the substrates and the inhibitors may derive from (a) the greater spacing between the binding and reacting sites and the greater conformational freedom in the inhibitor conferred by the additional methylene group and (b) the different stereoelectronic requirements for disulphide cleavage as against amide bond cleavage. A further consequence of the relaxation of the constraints on ligand binding is that it allows relative improvement in the binding of the D-

Table 3. Effects on reactivities and difference kinetic specificity energies ($\Delta\Delta G_{KS}$) of successive additions of binding opportunities in the reactions of papain with substrate-derived 2-pyridyl disulphides

Structural change in reactivity probe	Potential binding features		Reaction with maximal contribution of transition state containing hydrogen bond from (His-159)-Im ⁺ H to leaving group		Reaction via anionic transition state lacking hydrogen bond to leaving group	
	Existing	Additional	Fold increase in reactivity	$\Delta\Delta G_{KS}$ (kJ·mol ⁻¹)	Fold increase in reactivity	$\Delta\Delta G_{KS}$ (kJ·mol ⁻¹)
<i>n</i> -Propyl-S-S-2-Py → (VI)	None	(P ₂) > C=O	7.3	5.0	9.2	5.4
(VI) → (V)	(P ₂) > C=O	(P ₁)-N-H	5.1	4.2	1.2	0.4
(V) → (II)	(P ₂) > C=O; (P ₁)-N-H	<i>N</i> -Ac-D-Phe	6.8	4.6	0.8	-0.8
(V) → (I)	(P ₂) > C=O; (P ₁)-N-H	<i>N</i> -Ac-L-Phe	207	13.4	30	8.4
(VI) → (IV)	(P ₂) > C=O	<i>N</i> -Ac-D-Phe	25	7.9	1.3	0.8
(VI) → (III)	(P ₂) > C=O	<i>N</i> -Ac-L-Phe	207	13.4	7.0	5.0
(IV) → (II)	(P ₂) > C=O; <i>N</i> -Ac-D-Phe	(P ₁)-N-H	1.4	0.8	0.7	-0.8
(III) → (I)	(P ₂) > C=O; <i>N</i> -Ac-L-Phe	(P ₁)-N-H	5.1	4.2	5.2	4.2

enantiomer in the S₂ subsite by a small rotation about the long axis of the molecule (counterclockwise when viewed from the leaving-group end). This rotation moves the phenylalanyl side chain away from the solvent interface and into the cleft, and requires only a small perturbation of the bridging hydrogen bonds. This rotation is opposite in direction to the rotational adjustment needed to improve catalytic-site alignment for the Ac-Phe-Gly 4-nitroanilide substrates.

Removal of the opportunity for formation of the (P₁)-N-H...O=C < (Asp-158) hydrogen bond by using the ester analogues [compounds (III) and (IV)] (Schemes 2*b* and 2*d*) is predicted to facilitate the rotational adjustment discussed above with a consequent decrease in the value of *I*_{ss}. Another factor that needs to be considered, however, is the consequence for binding in the S₂ subsite of the binding interaction in the catalytic site. This appears to be important in that the data in Table 3 suggest that the *N*-Ac-D-Phe side chain has very little effect on reactivity unless the hydrogen bond from (His-159)-Im⁺H to the leaving group exists, irrespective of whether the (P₁)-N-H...O=C < (Asp-158) hydrogen bond exists [(V) → (II)] or not [(VI) → (IV)]. The apparent dependence of P₂-S₂ stereochemical selectivity on the hydrogen bond to the leaving group may account for the low selectivity reported by Hanzlik *et al.* (1991) for the papain-catalysed hydrolyses of the enantiomeric *N*-acetyl-Phe-Gly-ONp esters (see Patel *et al.*, 1992).

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