A re-appraisal of the structural basis of stereochemical recognition in papain

Insensitivity of binding-site–catalytic-site signalling to P₂-chirality in a time-dependent inhibition

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1. 2-(*N'*-Acetyl-D-phenylalanyl-amino)ethyl 2'-pyridyl disulphide (compound I) [m.p. 123–124 °C; $[\alpha]_D^{20} - 7.1^\circ$ (*c* 0.042 in methanol)] was synthesized, and 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 of papain (EC 3.4.22.2), together with existing kinetic data for the analogous reaction of the L-enantiomer (compound II), were used to evaluate the consequences for transition-state geometry of the difference in chirality at the P₂ position of the probe molecule. 2. The kinetic data suggest that the D-enantiomer binds approx. 40-fold less tightly to papain than the L-enantiomer but that the binding-site–catalytic-site signalling that results in a (His-159)–Im⁺–H-assisted transition state occurs equally effectively in the interaction of the former probe as in that of the latter. This results in pH–*k* profiles for the reactions of both enantiomers each characterized by four macroscopic p*K*_a values (3.7–3.9, 4.1–4.3, 7.9–8.3 and 9.4–9.5) in which *k* is maximal at pH approx. 6 where the –Im⁺–H-assisted transition state is most fully developed. 3. Model building indicates that both enantiomers can bind to papain such that the phenyl ring of the *N*-acetylphenylalanyl group makes hydrophobic contacts in the binding pocket of the S₂ subsite with preservation of the three hydrogen-bonding interactions involving the substrate analogue reagent and (Asp-158)C=O, (Gly-66)C=O, and (Gly-66)N–H of papain. Earlier predictions that binding of *N*-acyl-D-phenylalanine derivatives to papain would be prevented on steric grounds [Berger & Schechter (1970) *Philos. Trans. R. Soc. London B* **257**, 249–264; Lowe & Yuthavong (1971) *Biochem. J.* **124**, 107–115; Lowe (1976) *Tetrahedron* **32**, 291–302] were based on assumed models that are not consistent with the X-ray-diffraction data for papain inhibited by alkylation of Cys-25 with *N*-benzyloxycarbonyl-Phe-Ala-chloromethane [Drenth, Kalk & Swen (1976) *Biochemistry* **15**, 3731–3738]. 4. The possibility that the kinetic expression of P₂–S₂ stereospecificity may depend on the nature of the chemistry occurring in the catalytic site of papain is discussed. 5. The Tyr-61/Tyr-67 pair is proposed as a possible candidate for one of the two kinetically influential p*K*_a values (approx. 8 or 9.5) that characterize the alkaline limbs of the pH–*k* profiles for the reactions of papain with compounds (I) and (II) and of the pH–(*k*_{cat.}/*K*_m) profile for papain-catalysed hydrolysis.

INTRODUCTION

The ways in which catalytic-site chemistry may depend upon specific binding interactions involving non-scissile parts of substrates are not well understood, and the cysteine proteinases provide opportunities for their study by kinetic methods. Papain, the best-characterized member of the cysteine proteinase family both structurally and mechanistically [see Baker & Drenth (1987), Brocklehurst (1987) and Brocklehurst *et al.* (1987*b*) for reviews, and Cohen *et al.* (1986), Bode *et al.* (1988), Lindahl *et al.* (1988), Yabe *et al.* (1988), Varughese *et al.* (1989), Storer *et al.* (1989) and Vernet *et al.* (1989) for recent crystallographic, spectroscopic and molecular-biological studies that further illuminate ligand binding], is particularly valuable in this respect because of the sensitive way in which the reactivity of its catalytic site responds to a combination of hydrogen-bonding and hydrophobic effects in its extended binding site.

The relative dispositions of the (Cys-25)S[–] and (His-159)–Im⁺–H catalytic-site side chains of papain

with respect to each other (see Pullman *et al.*, 1989) and to electrophilic centre and leaving group, and their perturbations by binding interactions, can be assessed by using substrate-derived 2-pyridyl disulphides as reactivity probes (see Brocklehurst *et al.*, 1988*a,b*; Kowlessur *et al.*, 1989*a,b*). Binding interactions that permit hydrogen-bonding of –Im⁺–H with the N atom of the 2-mercapto-pyridine leaving group simultaneously with the attack of S[–] at the electrophilic S atom produce a striking maximum in the pH–second-order rate constant (*k*) profile at approx. pH 6. This contrasts markedly with the situation that obtains when the necessary binding interactions [notably involving (P₂)C=O and the P₂(L-Phe) side chain] are absent. Then, there is a rate minimum at pH 6–7, and a rate maximum at pH 3–4 produced by activation of the probe molecule by formal hydronation of the pyridine N atom rather than by its acceptance of a hydrogen bond from (His-159)–Im⁺–H.

P₂–S₂ hydrophobic contacts play a particularly important role not only in the binding of ligands to papain

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but also in providing the transition-state geometry required for the $\text{Im}^+\text{-H}$ -assisted reaction. Although in its interactions with peptide substrates and with some inhibitors papain usually exerts high specificity for L-amino acid residues at the P_2 position (Lowe & Yuthavong, 1971; Lowe, 1976; Drenth *et al.*, 1976), this phenomenon appears to depend upon the nature of the chemical events occurring remote from the S_2 subsite in the catalytic site. Thus Mackenzie *et al.* (1986) reported that both *N*-acetyl-L- and *N*-acetyl-D-phenylalanyl-glycinal successfully form thiohemiacetals with papain and that the D-enantiomer is bound to papain only 5-fold less tightly than the L-enantiomer (presumably by binding in the S_2 subsite in each case). In view of this finding, it was decided to study the reaction of papain with the D-enantiomer of 2-(*N'*-acetyl-L-phenylalanyl-amino)ethyl 2'-pyridyl disulphide (compound I), i.e. compound (II).

In the present work the objective was to compare the binding to papain of the enantiomeric pyridyl disulphides, compounds (I) and (II), and to ascertain the extent to which the binding mode of the D-enantiomer (compound II) provides for the disposition of (Cys-25)- S^- , (His-159)- $\text{Im}^+\text{-H}$, electrophilic S and leaving-group pyridyl N atom necessary for the transition-state geometry of the $\text{Im}^+\text{-H}$ -assisted reaction. To this end, the synthesis and characterization of 2-(*N'*-acetyl-D-phenylalanyl-amino)-ethyl 2'-pyridyl disulphide (compound II) and the pH-dependent kinetics of its reaction with papain are reported. Although papain reacts with the D-enantiomer (compound II) about 30–60-fold more slowly than with the L-enantiomer (compound I), this occurs in all reactive hydronic states and the shape of the pH-*k* profile characteristic of efficient signalling from binding area to catalytic site is essentially preserved. Possible binding modes that could account for the approximately equally effective signalling in complexes of papain with each of the enantiomeric peptidyl 2-pyridyl disulphides are identified by model building, and the dependence of the expression of specificity in $\text{S}_2\text{-P}_2$ interaction on the nature of the chemistry required in the catalytic site of papain is discussed. Conclusions about the stereochemistry of $\text{P}_2\text{-S}_2$ binding reached by Berger & Schechter (1970) and by Lowe & Yuthavong (1971) and Lowe (1976), which rely on assumed models of papain-ligand complexes that are not in accord with the high-resolution X-ray-diffraction data (Drenth *et al.*, 1976; Kamphuis *et al.*, 1984), are re-evaluated.

MATERIALS AND METHODS

Materials

Papain contaminated only by its oxidation products was isolated as described by Baines & Brocklehurst (1979), checked for the absence of chymopapain contaminants (Baines & Brocklehurst, 1978) and produced in fully active form 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-amino)ethyl 2'-pyridyl disulphide (compound II) was synthesized by a route analogous to that reported for the L-enantiomer (Brocklehurst *et al.*, 1987a), with *N*-t-butyloxycarbonyl-D-phenylalanine *N*-hydroxysuccinimide ester (Sigma Chemical Co.) as starting material. The product was recrystallized by dissolution in ethyl acetate followed by addition of hexane to turbidity and storage at 4 °C, and then had m.p. 123–124 °C and $[\alpha]_D^{20} -7.1^\circ$ (*c* 0.042 in methanol) (Found: C, 57.60; H, 5.56; N, 11.12; $\text{C}_{17}\text{H}_{19}\text{N}_3\text{O}_2\text{S}_2$ requires C, 57.6; H, 5.6; N, 11.2 %). An aqueous solution of a sample produced the predicted yield of pyridine-2-thione consequent upon thiolysis with 2-mercaptoethanol at pH 7, deduced by spectral analysis at 343 nm ($\epsilon_{343} = 8.08 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$; Stuchbury *et al.*, 1975). The i.r. spectra of the L- and D-enantiomers (compounds I and II respectively) were identical. The characteristics of the L-enantiomer are: m.p. 123 °C; $[\alpha]_D^{20} +7.0^\circ$ (*c* 0.043 in methanol). When these characteristics were reported (Brocklehurst *et al.*, 1987a), the value of *c* was wrongly printed as 0.43.

Kinetics

All reactions were studied at 25 °C and 1/0.1 in solutions containing 1 mM-EDTA with Dionex stopped-flow equipment connected to a Tektronix 5103N oscilloscope and interfaced with a Commodore 4032 microcomputer. Some kinetic runs in each set were recorded by photographing the oscilloscope screen, and conventional analysis of these records always provided values of rate constants in good agreement with those produced by the on-line microcomputer. Release of pyridine-2-thione consequent on reaction of the enzyme thiol group with the 2-pyridyl disulphide probe reagents was monitored at 343 nm and quantified by using $\epsilon_{343} = 8.0 \times 10^3 / (1 + K/[\text{H}^+]) \text{ M}^{-1} \cdot \text{cm}^{-1}$, where $\text{p}K = 9.8$ (Brocklehurst & Little, 1973; Stuchbury *et al.*, 1975). The buffers were as described by Brocklehurst *et al.* (1987a). Most reactions were carried out under pseudo-first-order conditions with [enzyme] = 3 μM and [compound (II)] = 30–40 μM in the mixing chamber of the stopped-flow machine. Linear first-order logarithmic plots provided k_{obs} , and at pH 3.4, 6.0 and 9.15 linear increase in [compound (II)] up to 70 μM confirmed that under the conditions of concentration used the reactions obeyed overall second-order kinetics. Values of second-order rate constants (*k*) were calculated from $k = k_{\text{obs}}/[\text{compound (II)}]$. Some kinetic runs at pH values about 6 were carried out also under equimolar second-order conditions with [enzyme] = [compound (II)] = 3–4 μM . The absorbance changes produced were those predicted for these concentrations and the reaction stoichiometry, and the values of *k* obtained from conventional linear second-order plots were closely similar to those obtained for reactions carried out under pseudo-first-order conditions. These findings preclude the possibility that reaction is with contaminant L-enantiomer rather than with the D-enantiomer. The pH-dependence of *k* and of the individual contributions from the various hydronic states [the International Union of Pure and Applied Chemistry (1986) suggests that the ion H^+ be called the hydron when an individual isotope is not being specified] was evaluated by using the eight-parameter (four- $\text{p}K_a$) rate

equation (eqn. 1) (Brocklehurst & Brocklehurst, 1988) and a BBC model B microcomputer fitted with a 6502 second processor:

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

The pH- k data and the theoretical lines were then displayed (as in Fig. 1) by using a Compaq Deskpro 386/20e PC and a Hewlett Packard Colour Pro Plotter.

RESULTS AND DISCUSSION

Comparison of the effects of D- and L-phenylalanine side chains at the P₂-position in 2-pyridyl disulphide probes on the pH-dependence characteristics of the rates of their reactions with papain

The pH- k profile for the reaction of papain with 2-(*N*-acetyl-D-phenylalanyl-amino)ethyl 2'-pyridyl disulphide (compound II) is shown in Fig. 1(a). The close similarity in the shape of this profile and that for the reaction of papain with the L-enantiomer (compound I) are demonstrated in Figs. 1(a) and 1(b) and by the ratios of the pH-independent rate constants for individual hydronic states in Table 1. If the degree of development of the rate maximum at pH 6 ($\tilde{k}_{\text{XH}_2}/\tilde{k}_{\text{X}}$) is taken as a measure of signalling from binding areas to catalytic site to provide for an -Im⁺-H-assisted transition state (see Kowlessur *et al.*, 1989a), it is clear that the signalling is insensitive to the stereochemistry of the phenylalanine residue at position P₂. This insensitivity was surprising in view of the reports that papain exerts marked specificity for L-amino acid residues at the P₂ position as well as at the P₁ position of peptide substrates (see, e.g., Lowe, 1976). If the difference in reactivity of the uncomplicated thiolate anion of papain towards the enantiomeric probes (i.e. in the X-state at high pH) is taken to arise principally from differences in binding (see Brocklehurst *et al.*, 1987a, 1988b), the L-enantiomer is deduced to bind approx. 40 times more tightly than the D-enantiomer. The reactivity ratios in other hydronic states suggest that the transition-state geometry for the -Im⁺-H-assisted reaction is achieved about equally well when either enantiomeric probe is bound to papain.

Stereochemistry of ligand binding to the S₂ subsite of papain and its consequences for catalytic-site chemistry

Before structural interpretation of the kinetic data in Fig. 1 and Table 1 is presented, it is useful to consider the evidence that has been used previously to infer stereospecificity of papain for L-chirality at position P₂. In particular it is necessary to explain (a) how the binding mode for substances with an L-phenylalanine residue at position P₂ assumed by Lowe & Yuthavong (1971) and by Lowe (1976), which is an important part of the argument in favour of high chiral specificity in the S₂ subsite, is not in accord with the crystallographic evidence, and (b) how, when a binding mode that is consistent with the X-ray-diffraction data is considered, it is clear that either enantiomeric ligand

with a phenylalanine residue at the P₂ position can be accommodated in the active centre of papain.

Fig. 1. pH-dependence of k for the reactions of papain with (a) 2-(*N*-acetyl-D-phenylalanyl-amino)ethyl 2'-pyridyl disulphide (II) and (b) its L-enantiomer (I) at 25 °C and / 0.1 in aqueous buffers

The points in (a) are experimental and the continuous lines in (a) and (b) are theoretical for eqn. (1), with values of $\tilde{k}$ in $\text{M}^{-1} \cdot \text{s}^{-1}$. The various values of $\tilde{k}$ and $\text{p}K_{\text{a}}$ determined for (a) in the present work are as follows, with those for (b) determined by Brocklehurst *et al.* (1988b) in parentheses: $\tilde{k}_{\text{XH}_3} = 1.0 \times 10^5$ (3.0×10^6), $\tilde{k}_{\text{XH}_2} = 2.0 \times 10^5$ (6.1×10^6), $\tilde{k}_{\text{XH}} = 4.0 \times 10^4$ (2.3×10^6), $\tilde{k}_{\text{X}} = 1.5 \times 10^4$ (6.0×10^5), $\text{p}K_{\text{XH}_4} = 3.7$ (3.9), $\text{p}K_{\text{XH}_3} = 4.3$ (4.1), $\text{p}K_{\text{XH}_2} = 7.9$ (8.25), $\text{p}K_{\text{XH}} = 9.4$ (9.5); broken lines A-D correspond to contributions to k of the individual hydronic states of the reaction, which are provided by the terms in eqn. (1) associated with $\tilde{k}_{\text{XH}_3}$ - $\tilde{k}_{\text{X}}$ respectively; the arrows in (b) define the pH range over which data points were collected.

Scheme 1. Diagram showing postulated binding interactions between papain and (a) 2-(N'-acetyl-L-phenylalanyl-amino)ethyl 2'-pyridyl disulphide (I) and (b) its D-enantiomer (II)

The drawings each show a view looking into the active-centre cleft towards the centre of the 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, e.g. in hydrogen bonds, have been exaggerated for clarity. The placing of enzyme side chains and backbone groups is not topographically correct, but it shows their relative dispositions around the cleft and their approximate spatial relationships to the ligand. The structural features of the papain-ligand complex in and around the S_2 subsite are analogous to those deduced by Drenth *et al.* (1976) from X-ray-crystallographic studies on papain-inhibitor combinations such as papain alkylated on S_{17} of Cys-25 by reaction with the terminal methylene group of *N*-benzyloxycarbonyl-L-Phe-L-Ala-CH₂Cl. The peptide between P_1 and P_2 lies near the extended piece of chain comprising residues 65-67 of the wall of the active-centre cleft such that the (P_2) -N-H and (P_2) -C=O groups can hydrogen-bond with the backbone C=O and N-H groups respectively of Gly-66 and the (P_1) -N-H group is directed towards the backbone C=O group of Asp-158 on the opposite wall of the cleft. In the alkylated papain derivative studied by Drenth *et al.* (1976) and, by analogy, in the papain-L-enantiomer complex shown in (a), the (P_2) - α -H atom projects out of the protein and the α -benzyl side chain is accommodated such that C_{α} lies between the side chains of Pro-68 and Ala-160, and beyond it the phenyl ring is adjacent to the side chains of Val-133 and Val-157, which

Table 1. Characteristics of the reactions at 25 °C and 1/0.1 of the catalytic-site thiol group of papain with the enantiomeric 2-(*N*-acetylphenylalanylaminomethyl) 2'-pyridyl disulphides (compounds I and II) in four reactive hydronic states designated X–XH₃ to indicate their stoichiometries in hydrons relative to that of the unreactive hydronic state XH₄ approached at low pH

	Macroscopic pK _a values				pH-independent rate constants (M ⁻¹ ·s ⁻¹)				$\frac{\tilde{k}_{\text{XH}_3}}{\tilde{k}_{\text{X}}}$	$\frac{\tilde{k}_{\text{XH}_2}}{\tilde{k}_{\text{X}}}$	$\frac{\tilde{k}_{\text{X}}}{\tilde{k}_{\text{XH}}}$
	pK _{XH₄}	pK _{XH₃}	pK _{XH₂}	pK _{XH}	10 ⁻⁶ × $\tilde{k}_{\text{XH}_3}$	10 ⁻⁵ × $\tilde{k}_{\text{XH}_2}$	10 ⁻⁵ × $\tilde{k}_{\text{XH}}$	10 ⁻⁵ × $\tilde{k}_{\text{X}}$	$\frac{\tilde{k}_{\text{XH}_3}}{\tilde{k}_{\text{X}}}$	$\frac{\tilde{k}_{\text{XH}_2}}{\tilde{k}_{\text{X}}}$	$\frac{\tilde{k}_{\text{X}}}{\tilde{k}_{\text{XH}}}$
L-Enantiomer*	3.9	4.1	8.25	9.5	30	61	23	6	5	10	4
D-Enantiomer	3.7	4.3	7.9*	9.4	1	2	0.4	0.15	7	13	3

* Data from Brocklehurst *et al.* (1988b); the ratios of reactivity towards the enantiomeric probes in the various states are: XH₃, $\tilde{k}_{\text{L}}/\tilde{k}_{\text{D}} = 30$; XH₂, $\tilde{k}_{\text{L}}/\tilde{k}_{\text{D}} = 31$; XH, $\tilde{k}_{\text{L}}/\tilde{k}_{\text{D}} = 58$; X, $\tilde{k}_{\text{L}}/\tilde{k}_{\text{D}} = 40$.

Berger & Schechter (1970) measured rates of hydrolysis of polyalanine peptides containing one or more D-alanine residues. Appreciable rates of hydrolysis (with cleavage sites indicated by †) were found only where the S₂, S₁ and S₁' subsites could be occupied by L-alanine residues, as, for example, in (Ala)₅(DLL†LL) and in (Ala)₅(LL†L†LD). They observed also that peptides in which L-phenylalanine is the third or further residue from the C-terminus are good substrates for papain, the scissile bond being always that linking the next two residues towards the C-terminus, i.e. L-Phe-Xaa†Yaa.

The indications from the work referred to above are that papain exerts an essentially absolute stereospecificity for L-amino acid residues (particularly L-phenylalanine) at position P₂, although there is some evidence in the early literature on papain-catalysed synthesis of anilides (Behrens *et al.*, 1940) and phenylhydrazides (Bennett & Niemann, 1948, 1950; Schuller & Niemann, 1951) that this might not be the case. In the present work we have shown not only that both enantiomeric 2-pyridyl disulphides containing *N*-acetylphenylalanine residues approximately in the P₂ position (compounds I and II) are capable of interacting with papain, but that they appear to provide approximately equally for the binding-site-catalytic-site signalling that results in the (His-159)–Im⁺–H-assisted transition state. This finding supports the conclusion of Mackenzie *et al.* (1986) that an *N*-acetyl-D-phenylalanine side chain is capable of binding in the S₂ subsite of papain, and necessitates reconsideration of some of the conclusions of Berger & Schechter (1970), Lowe & Yuthavong (1971) and Lowe (1976).

One difficulty (for others see also Fruton, 1982) with the conclusion reached by Berger & Schechter (1970) (see also Schechter & Berger, 1967, 1968), namely that strict stereospecificity for L-amino acid residues is exerted by the S₂ subsite of papain, derives from their assumed model for the enzyme–substrate complex. The suggested binding mode of the heptapeptide (L-Ala)₂–L-Phe–(L-Ala)₄ was such that the substrate is bent back on itself and was assumed to make the following hydrogen bonds that are now known, from the X-ray-diffraction

data of Drenth *et al.* (1976), to be improbable: (P₁)>C=O...H–⁺Im–(His-159), (P₂)>C=O...H–N–(Trp-177), (P₃)>C=O...H–NH–(Gln 19), (P₄)>C=O...H–N–(Gly-23) and (P₂')–N–H...O=C<(Asp-158). In terms of this model the affinity of papain for a hydrophobic side chain at position P₂ is attributed to its contact with the indole side chain of Trp-177 (cf. Scheme 1, which is based on the crystallographic data of Drenth *et al.*, 1976), and the strict stereospecificity of the S₁, S₂ and S₁' subsites could be accounted for as follows. When the relevant hydrogen bonds are assumed to exist, the α-hydrogen atoms of residues in positions P₁, P₂ and P₁' (each in the L-configuration) point toward protein. Since the situation would be reversed with the residues in the D-configuration, steric effects would preclude formation of the hydrogen bonds. Lowe & Yuthavong (1971) corrected many of the glaring errors in the substrate-binding model proposed by Berger & Schechter (1970) and suggested that the specificity of papain for L-amino acid residues at the P₂ position could be accounted for by the binding of the P₁–P₂ peptide bond and the location of the P₂ side chain in the S₂ subsite. Their inspection of molecular models suggested that the α-CH of an L-amino acid residue at the P₂ position points into the body of the protein and that there would be insufficient space to accommodate the *N*-acylamino group of a D-amino acid residue, a view subsequently restated by Lowe (1976). The apparent validity of this argument appears to depend on an incorrect assumption about binding modes made by Lowe & Yuthavong (1971) before the crystallographic *et al.* (1976) became available. Thus if, as Lowe & Yuthavong (1971) suggested, the (P₂)–N–H did donate a hydrogen bond to the (Asp-158)–CO₂[–], then for the L-configuration (P₂)–α-CH would point into the protein and the side chain out of the protein. In the binding modes revealed by the X-ray-diffraction data for papain inhibited by reaction with *N*-benzyloxycarbonyl-L-Phe-L-Ala-CH₂Cl (Drenth *et al.*, 1976), however, (P₂)–N–H donates a hydrogen bond to (Gly-66)>C=O (see Scheme 1) rather than to Asp-158. For an L-amino acid residue at position P₂, therefore, α-CH points out of the protein

with Tyr-67, Trp-69 and Phe-207 form the hydrophobic binding pocket. The pyridyl N atom is shown in hydrogen-bonding interaction with the imidazolium side chain of His-159, which is considered to be responsible for the striking rate maximum at pH approx. 6 (Fig. 1b). In the papain–D-enantiomer complex shown in (b) all four hydrogen bonds can be maintained, and, even though the (P₂)–α-H atom projects into the protein, the α-benzyl side chain can still be accommodated either close to the region in the S₂ subsite occupied by this side chain in the complex with the L-enantiomer (a) or, by rotation about C_(α)–C_(β), with the phenyl ring close to Tyr-67. The rotated position of the phenyl ring is indicated by broken lines and the phenyl ring points away from the centre of the papain molecule and towards the L domain.

and the $C_{(\alpha)}-C_{(\beta)}$ bond points into it rather than the other way round as suggested previously (Lowe, 1976) even when the correct hydrogen-bonding partner for $(P_2)-N-H$ had been identified. Even in the binding mode now perceived with $\alpha-CH$ pointing out of the protein, the phenyl ring of the L-phenylalanine residue at position P_2 can fit into the hydrophobic pocket of the S_2 subsite, making van der Waals contacts with Val-133, Ala-160 and Pro-68, because of the 109.5° bond angle at $C_{(\beta)}$. Inspection of the molecular model of papain suggests that, for a ligand with a D-phenylalanine residue at position P_2 , the phenyl ring could either adopt a rather similar position to its location for the L-enantiomer or alternatively rotate to make hydrophobic contacts with Tyr-61 and Tyr-67. The transformation of the binding mode suggested by Lowe & Yuthavong (1971) into that revealed by Drenth *et al.* (1976) involves rotation about the $(P_2) C_{(\alpha)}-CO$ bond, and the conceptual conversion of the L-enantiomer into the D-enantiomer merely involves transposition of the H- and $-CH_2Ph$ substituents at $(P_2) C_{(\alpha)}$. This does not appear to require substantial perturbation of the conformation and hydrogen-bonding interactions of the remainder of the ligand (substrate or inhibitor) with the protein. The conclusions about ligand binding to papain reached in the present work agree with those reached by Mackenzie *et al.* (1986) in that the S_2 subsite does not appear to have absolute specificity for N-acetylated amino acid residues. They differ, however, in one respect. Mackenzie *et al.* (1986) suggested that for the D-enantiomer it is necessary to rotate the N-acetyl group about the phenylalanine $C_\alpha-N$ bond such that it projects into solvent in order that it may adopt a non-sterically hindered position. The rotation suggested would move the phenylalanine N-H bond out of the plane necessary for optimal hydrogen-bonding to Gly-66. In the present work, model building suggests that non-sterically hindered positions for both enantiomers may be attained without this rotation. Suggested binding modes for the enantiomeric 2-pyridyl disulphide probes (compounds I and II) are shown in Scheme 1. In each case the hydrophobic P_2-S_2 contacts and the transcleft hydrogen bonds necessary for provision of the $(His-159)-Im^+-H$ -assisted transition state can be achieved. It may be that particular chemistries occurring in the catalytic site such as $(His-159)-Im^+-H$ -assisted substitution at acyl C (as in the catalysed hydrolysis of substrates) and at other electrophilic centres such as S (as in the 2-pyridyl disulphide inhibitors) have different sensitivities to more subtle aspects of the binding process than are readily revealed by model building. Evidence that binding modes and catalytic-site chemistries may be interdependent is provided by Fruton's work with fluorescent peptides (Henes *et al.*, 1979). Thus papain is strongly inhibited by the peptidyl aldehyde mansyl-Gly-Gly-L-Phe-glycinal, which carries the mansyl fluorescent reporter group and where glycinal corresponds to the P_1 residue of a substrate. The environment of the N-terminal fluorescent probe in the enzyme-inhibitor combination appears to be quite different from that of the same probe attached to the P_4 residue of the sensitive papain substrate mansyl-Gly-Gly-Val-Glu-Leu-Gly, where the Glu-Leu bond is the site of cleavage. Thus, whereas there is a marked enhancement of the mansyl fluorescence when papain binds the substrate (Mattis & Fruton, 1976), no such enhancement occurs when papain reacts with the glycinal (Henes *et al.*, 1979).

Origin of the pH-dependence of the reaction of papain with the enantiomeric 2-(N-acetylphenylalanyl-amino)-ethyl 2'-pyridyl disulphides (compounds I and II)

The pH-dependent behaviour of both the reactivity of the catalytic-site thiol group of papain and its catalytic activity ($k_{cat.}/K_m$) contrasts markedly with that of the analogous properties of the closely similar enzyme actinidin (Brocklehurst *et al.*, 1988b). In alkaline media it is only in the reactions of papain that a kinetically influential pK_a with a value approx. 8 is observed when the 2-pyridyl disulphide probe contains a P_1-P_2 amide bond (as it is for $k_{cat.}/K_m$) in addition to the pK_a approx. 9.5, which is the only pK_a value (> 7) that affects reactivity towards probes lacking the P_1-P_2 amide bond. A structural interpretation of these marked differences in behaviour of the two enzymes requires identification of a candidate for the additional hydronic dissociation in papain that is without analogue in actinidin. One candidate remote from the active centre of papain (His-81), for which the sequence-aligned residue in actinidin is Asn-84, was discussed by Brocklehurst *et al.* (1988b). Another possible candidate, this one in close proximity to the principal binding area of papain, is one or other of the tyrosine residues (Tyr-61 or Tyr-67, which could form a hydrogen-bonded pair; see Scheme 1). In actinidin Tyr-67 is conserved, but the sequence-aligned residue analogous to Tyr-61 in papain is Arg-61 in actinidin. The widening of the binding cleft of papain consequent upon inhibitor binding was shown to be most marked in the region near Tyr-61 and Tyr-67 (Drenth *et al.*, 1976). If hydronic dissociation of the imidazolium cation of His-159 in the catalytic-site interactive system of papain is determined principally by the macroscopic pK_a approx. 8, rather than by the pK_a approx. 9.5, the reactivity towards probes (I) and (II) and $k_{cat.}/K_m$ would be increased by hydronic dissociation across pK_a 9.5. Conversely, if His-159 is associated with pK_a 9.5, the effect of hydronic dissociation from the modulating group of pK_a 8 would be to decrease both reactivity and catalytic activity. Assignment of the pK_a 8 to His-159 would mean that the pH-dependent reactivity in alkaline media of the papain thiol group towards 2-pyridyl disulphide probes that lack the P_1-P_2 amide bond (where the kinetically significant pK_a is 9.5; see Brocklehurst *et al.*, 1988b) would need to be attributed to a conformation change or binding effect rather than simply to the production of $-Im^-/S^-$ from $-Im^+-H/S^-$.

Concluding remarks

In the present work, evidence has been presented that N-acetylphenylalanyl derivatives can interact with papain such that the hydrophobic side chain occupies the S_2 subsite with retention of important hydrogen bonds for both the L- and D-enantiomers. Furthermore, in both cases the signalling that derives from the binding modes provides for transition-state geometries in which nucleophilic attack of the thiolate anion of Cys-25 is assisted by association of the imidazolium cation of His-159 with the leaving group. Suggestions in the literature that papain can make the binding contacts now considered essential for efficient binding-site-catalytic-site signalling only for the L-enantiomers of N-acylphenylalanine derivatives result from assumptions about papain-ligand binding modes that are not in accord with the high-resolution X-ray-diffraction data. The factors

governing the sensitivity with which binding modes are coupled with, and permit, particular catalytic-site chemistries remain to be established.

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