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# Variation in the $P_2$–$S_2$ stereochemical selectivity towards the enantiomeric *N*-acetylphenylalanylglycine 4-nitroanilides among the cysteine proteinases papain, ficin and actinidin

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1. Values of the kinetic specificity constant, $k_{cat.}/K_m$, for the hydrolysis of *N*-acetyl-L-phenylalanylglycine 4-nitroanilide (I) and of its D-enantiomer (II) catalysed by ficin (EC 3.4.22.3) and by actinidin (EC 3.4.22.14) at pH 6.0, *I* 0.1 mol/l, 8.3% (v/v) *NN*-dimethylformamide and 25 °C were determined by using initial-rate data with [S] $\ll K_m$ and weighted non-linear regression analysis as: for ficin, $(k_{cat.}/K_m)_L = 271 \pm 6$ $M^{-1} \cdot s^{-1}$, $(k_{cat.}/K_m)_D = 2.9 \pm 0.1$ $M^{-1} \cdot s^{-1}$, and for actinidin $(k_{cat.}/K_m)_L = 13.3 \pm 0.7$ $M^{-1} \cdot s^{-1}$, $(k_{cat}/K_m)_D = 0.34 \pm 0.01$ $M^{-1} \cdot s^{-1}$. 2. These data and analogous values for the corresponding reactions catalysed by papain (EC 3.4.22.2), $(k_{cat.}/K_m)_L = 2064 \pm 31$ $M^{-1} \cdot s^{-1}$, $(k_{cat.}/K_m)_D = 5.5 \pm 0.1$ $M^{-1} \cdot s^{-1}$, demonstrate marked variation in stereochemical selectivity for substrates (I) and (II) among the three cysteine proteinases with the following values for the index of stereochemical selectivity $I_{ss} = (k_{cat.}/K_m)_L/(k_{cat.}/K_m)_D$: for papain, 375; for ficin 93; for actinidin 39. 3. Model building suggests ways in which, for the papain-catalysed reactions, binding interactions involving the extended acyl groups of the substrates may need to change as the reaction proceeds from adsorptive complex (ES) to tetrahedral intermediate (THI) before its rate-determining, general acid-catalysed collapse to acylenzyme intermediate. In particular, satisfactory alignment in the catalytic site at the THI stage of the acylation process appears to demand rotation of the substrate moiety about its long axis. 4. The different consequences of this rotation for the L- and D-enantiomers suggest that for closely related systems the greater the extent of this rotational adjustment the greater would be the value of $I_{ss}$. 5. For the actinidin–substrate combinations, model building suggests that even at the ES complex stage of catalysis it is not possible to approach optimized $P_2$–$S_2$ contacts and the three hydrogen-bonding interactions deduced for papain–ligand complexes in the absence of significant movement of protein conformation. Possible binding modes in which some of the interactions deduced for papain are relaxed are discussed. Consideration of postulated binding modes in the various transition states is shown to account for the order of reactivity reflected in values $k_{cat.}/K_m$ for the four reactions involving papain (Pap) and actinidin (Act) with the L- and D-enantiomeric substrates: Pap–L $\gg$ Act–L > Pap–D $\gg$ Act–D. 6. The intermediate position between papain and actinidin occupied by ficin in stereochemical discrimination between substrates (I) and (II) suggests that structural and structure-related kinetic studies on this enzyme may contribute substantially to the developing picture of structure–function relationships in the cysteine proteinase family.

## INTRODUCTION

Molecular recognition [see Roberts (1989) for reviews] is often taken to refer to the selective binding of one molecule with another by making use of a combination of some or all of four types of non-covalent interaction: electrostatic, hydrogen-bonding, van der Waals and hydrophobic, the latter arising mainly from a favourable desolvation effect of non-polar residues (Kollman, 1989). 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 catalytic-site chemistry. This was emphasized by results from early site-directed mutagenesis experiments (Knowles, 1987) where unexpected consequences of relatively minor structural changes were reported. For example, trypsin mutants designed to discriminate between lysine and arginine at $P_1$ were found to be much less active than the wild-type with dominant effects on $k_{cat.}$ rather than on $K_m$ (Craik *et al.*, 1985). Also, the extensive range of modifications in the binding areas of subtilisin produced a set of mutant enzymes for which effects on overall catalytic activity were not predictable, with $k_{cat.}$ values varying independently of $K_m$ values (Estell *et al.*, 1986; Wells *et al.*, 1987).

The cysteine proteinase family of enzymes is proving to be a valuable test-bed for exploring the coupling of different binding interactions with each other and with catalytic-site chemistry (Brocklehurst *et al.*, 1988*a*,*b*; Kowlessur *et al.*, 1989*a*,*b*, 1990; Templeton *et al.*, 1990; Berti *et al.*, 1991; Hanzlik *et al.*, 1991). The particular value of this enzyme family derives from the natural structural variation within it (see Brocklehurst, 1986, 1987; Brocklehurst *et al.*, 1987*b*, for reviews) and from the existence of a highly reactive nucleophilic centre ($-S^-$) in the common cysteine–histidine interactive system of the catalytic sites which has a nucleophilic role in the catalytic act. The latter permits the comparison of data from kinetics of catalysis with those from reactions with thiol-specific time-dependent inhibitors. These can be designed to involve single-step covalency change analogous to an individual step of the catalytic act, and to allow the characterization of both the uncomplicated thiolate anion and catalytically competent thiolate–imidazolium ion-pair states of the enzyme (Brocklehurst, 1982; Brocklehurst *et al.*, 1987*a*).

We recently identified possible binding modes in complexes of papain (EC 3.4.22.2) with enantiomeric *N*-acetylphenylalanine-containing ligands (Templeton *et al.*, 1990; Kowlessur *et al.*, 1990; cf. Lowe & Yuthavong, 1971*a*) that are in accord with the

Abbreviation used: THI, tetrahedral intermediate.

high-resolution X-ray diffraction data that are available for other papain–ligand combinations (Drenth *et al.*, 1976; Kamphuis *et al.*, 1984). We reported also for the first time the quantification of the $P_2$–$S_2$ stereochemical selectivity of papain towards substrates containing a hydrophobic occupant for the $S_2$ subsite and opportunities to participate in the array of hydrogen-bonding interactions postulated to be important for molecular recognition and catalytic competence (Kowlessur *et al.*, 1990). Stereochemical selectivity is an important characteristic of enzymes and involves both binding and catalytic-site chemistry. Previously, it had been assumed that papain exerted an essentially absolute specificity for ligands containing L-phenylalanine residues at $P_2$. The demonstration that stereochemical selectivity can be quantified in papain–ligand systems suggests the determination of the selectivity index $I_{ss} = (k_{cat.}/K_m)_L/(k_{cat.}/K_m)_D$ as a useful parameter in the study of related enzyme–ligand systems. Characterization of any variation in $I_{ss}$ with systematic change in ligand and variation in enzyme structure may contribute to our understanding of the coupling of binding interactions with each other and with catalytic-site chemistry. In the present work, we have compared the stereochemical selectivities of three cysteine proteinases, papain, ficin (EC 3.4.22.3) and actinidin (EC 3.4.22.14), for the enantiomeric *N*-acetylphenylalanylglycine 4-nitroanilides at pH 6.0. There are marked differences between the stereochemical selectivities of all three enzymes for these substrates. Those of papain and actinidin, which are particularly marked, are discussed in terms of their high-resolution crystal structures. The intermediate position between papain and actinidin occupied by ficin in its stereochemical selectivity suggests that structural and structure-related kinetic studies on this enzyme with reactivity probes designed to detect and characterize binding site–catalytic site coupling might contribute substantially to the analysis of patterns of structure–reactivity relationships for the cysteine proteinase family.

## MATERIALS AND METHODS

### Materials

**Enzymes.** The purification of actinidin and the determination of active-centre concentrations of both actinidin and ficin by titration with 2,2′-dipyridyl disulphide have been reported previously (see Malthouse & Brocklehurst, 1976; Brocklehurst *et al.*, 1981; Salih *et al.*, 1987). Ficin was the 2× crystallized product (*ficin glabrata*) of Sigma, Poole, Dorset, U.K. F.p.l.c. (Pharmacia LKB) analysis of this product and of the Sigma crude ficin powder on a Mono S HR5/5 column identifies the 2× crystallized product as (a) the major component of the crude powder and (b) ficin III (Malthouse & Brocklehurst, 1976). In preparation for a set of kinetic experiments, 0.5 ml of the suspension of 2× crystallized ficin was mixed with an equal volume of 40 mM-cysteine solution in sodium pyrophosphate buffer pH 8.0, *I* 0.3 mol/l and allowed to stand for 30 min at room temperature (approx. 22 °C) to convert any reversibly oxidized ficin 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 ficin 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 ficin for active-site titration with 2,2′-dipyridyl disulphide and approx. 20 initial-rate determinations with the 4-nitroanilide substrates (see below).

Molecular models of papain and actinidin based on the co-ordinates of Drenth *et al.* (1976) and Baker (1980, 1981) respectively were supplied by Labquip, Reading, Berks, U.K.

### Enantiomeric *N*-acetyl-Phe-Gly 4-nitroanilides

The synthesis of these compounds was described by Kowlessur *et al.* (1990).

**Kinetics.** Initial rates ($v$) of release of 4-nitroaniline from the enantiomeric 4-nitroanilide substrates at 25 °C in sodium phosphate buffer, pH 6.0, containing 8.3% (v/v) *NN*-dimethylformamide with [E] = 1–3 μM and [S] 70–180 μM for the D-enantiomer and 40–230 μM for the L-enantiomer in a 3 ml reaction volume were determined at 410 nm and quantified by using $\epsilon_{410} = 8.8 \times 10^3\ M^{-1} \cdot cm^{-1}$ (Erlanger *et al.*, 1961). Some runs were performed by using a Kontron Uvikon 810 spectrophotometer and others by using a Cary 1 spectrophotomer/IBMPS/2 microcomputer and monitor system coupled to an Epson FX/850 printer.

Closely similar results were obtained with each instrument. When the Uvikon 810 spectrophotometer was used, the following absorbance ranges and chart speeds were found to be appropriate: for actinidin and the L-enantiomer, 0–0.02 *A* and 1–2 $cm \cdot min^{-1}$; for actinidin and the D-enantiomer, 0–0.01 *A* and 0.2 and 0.5 $cm \cdot min^{-1}$; for ficin and the L-enantiomer, 0–0.05 *A* and 5 $cm \cdot min^{-1}$; for ficin and the D-enantiomer 0–0.01 *A* and 0.5 and 1 $cm \cdot min^{-1}$. When the Cary 1 spectrophotometer was used, data-collection times of 3 min and 5–10 min were used for ficin and the L-enantiomer and ficin and the D-enantiomer respectively. The linear progress curve was viewed on the monitor and the slope corresponding to the line of best fit according to the least-squares criterion was computed and used with $\epsilon_{410}$ to calculate $v$ in $mol \cdot l^{-1} \cdot s^{-1}$.

### Parameter evaluation

All kinetic runs were carried out under conditions of substrate concentration such that plots of $v$ against [S] were linear, i.e. with $[S] \ll K_m$, such that $(v/[E]) = (k_{cat.}/K_m)$ [S]. Kinetic data ([S], $v$ pairs) were fitted to this rate equation by using weighted non-linear-regression analysis using the AR computer program (1988 PC version) from the BMDP statistical software package (Dixon *et al.*, 1988) and a Compaq Deskpro 386/20e PC and displayed by using a Hewlett–Packard Colour Pro Plotter. Constant relative error was assumed with rates inversely proportional to $v^2$. The procedure provided best-fit values of $k_{cat.}/K_m$ and associated standard errors (see Wilkinson, 1961).

## RESULTS AND DISCUSSION

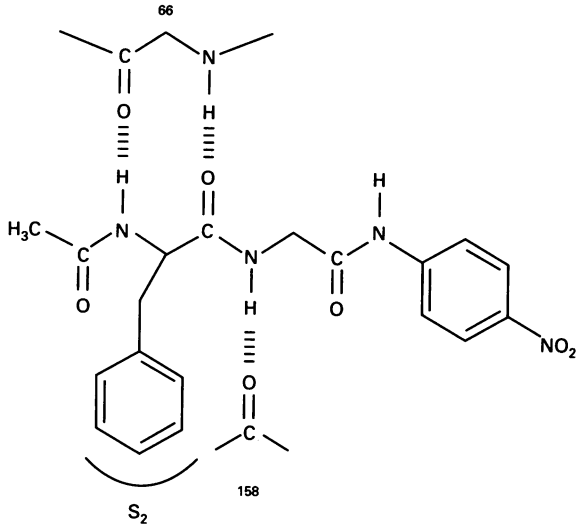
### The enzymes

Papain and actinidin are of particular interest because their three-dimensional structures are known from X-ray crystallographic studies (Drenth *et al.*, 1968, 1971, 1976; Baker, 1980, 1981; Kamphuis *et al.*, 1985; Baker & Drenth, 1987) and despite closely similar structures, they display considerable differences in behaviour (see Salih *et al.*, 1987; Brocklehurst *et al.*, 1988*a*; Kowlessur *et al.*, 1989*a*; Björk *et al.*, 1989; Björk & Ylinenjärvi, 1990).

The relatively little-studied cysteine proteinase, ficin (see Liener & Friedenson, 1970, and Glazer & Smith, 1971, for reviews), was of interest for the present study, despite the existence of very little structural information (see Husain & Lowe, 1970). The interest arose from the difference between the behaviour of its catalytic site and that of papain (Malthouse & Brocklehurst, 1976) and the intermediate position occupied by ficin, between papain and actinidin, in the characteristics of binding of chicken cystatin (Björk & Ylinenjärvi, 1990).

### The substrates

The enantiomeric substrates (I) and (II) (Scheme 1) were designed to provide a chromophoric product (4-nitroaniline) resulting from nucleophilic attack by the thiolate anion component of the Cys-25–His-159 ion-pair system of the cysteine proteinase catalytic site with assistance to leaving-group departure by hydron donation from (His-159)–Im$^+$H, and opportunities to engage in the following binding interactions in the $S_2$ subsite and $S_1$–$S_2$ intersubsite regions: $(P_2)$-NH ... O=C < (Gly-66), $(P_2)$ > C=O ... H-N-(Gly-66), $(P_2)$-$CH_2$Ph($S_2$), and $(P_1)$-NH ... O=C < (Asp-158). The hydrogen-bonding and $P_2$–$S_2$ hydrophobic interactions are the main specificity determinants for papain (see Drenth *et al.*, 1976; Asbóth *et al.*, 1988; Templeton *et al.*, 1990; Kowlessur *et al.*, 1990; Berti *et al.*, 1991) and some or all of them might be expected to exist also in complexes of substrates with the analogous enzymes ficin and actinidin.




**Scheme 1. Diagram showing the main binding interactions postulated between the enantiomeric *N*-acetyl-Phe-Gly 4-nitroanilides and the $S_1$–$S_2$ regions of papain**

The three hydrogen bonds shown involve the backbone carbonyl group of Asp-158 and the backbone carbonyl and amino groups of Gly-66; the benzyl side chain of the Phe residue of the substrate is accommodated within the hydrophobic $S_2$ subsite region; interactions within the catalytic site are not shown; see Scheme 2(*a*–*c*) for the postulated binding modes of both enantiomers at the tetrahedral intermediate (THI) stage of the reaction catalysed by papain and for the postulated binding mode of the D-enantiomer at the THI stage of the reaction catalysed by actinidin.

### Assessment of the influence of $P_2$ chirality on the effectiveness of the hydrolysis of the *N*-acetyl-Phe-Gly 4-nitroanilides catalysed by papain, ficin and actinidin

The assessment was made by comparing the values of $k_{cat.}/K_m$ at pH 6.0, which is in the pH region where $k_{cat.}/K_m$ is maximal for papain-catalysed hydrolysis (Lowe & Yuthavong, 1971*b*; Brocklehurst *et al.*, 1987*b*) and ficin-catalysed hydrolysis (Hollaway *et al.*, 1971) and close to maximal for actinidin-catalysed hydrolysis (Salih *et al.*, 1987). The usefulness in the context of the present type of study of $k_{cat.}/K_m$ as an index of kinetic specificity and enzyme effectiveness (developed by Bender & Kézdy, 1965; Brocklehurst *et al.*, 1968; Brot & Bender, 1969; Brocklehurst & Cornish-Bowden, 1976; Fersht, 1977; Brocklehurst, 1977) was summarized by Kowlessur *et al.* (1990).

The values of $k_{cat.}/K_m$ for the hydrolysis of the enantiomeric substrates catalysed by each of these three cysteine proteinases together with values of $I_{ss}$, the index of stereochemical selectivity, and the relative values of $(k_{cat.}/K_m)_L$, $(k_{cat.}/K_m)_D$, and $I_{ss}$ for the three enzymes are collected in Table 1.

The stereochemical selectivity exhibited in the catalysed hydrolysis of substrates (I) and (II) is much lower for actinidin ($I_{ss}$ = 39) than for papain ($I_{ss}$ = 375), with ficin occupying an intermediate position between the other two enzymes ($I_{ss}$ = 93). The approximately 10-fold difference between the stereochemical selectivity of papain and that of actinidin arises from a 155-fold difference in $(k_{cat.}/K_m)_L$ and a 16-fold difference in $(k_{cat.}/K_m)_D$. The analogous differences between papain and ficin are approximately 4-fold in $I_{ss}$, 8-fold in $(k_{cat.}/K_m)_L$ and 2-fold in $(k_{cat.}/K_m)_D$ and between ficin and actinidin, approximately 2-fold in $I_{ss}$, 20-fold in $(k_{cat.}/K_m)_L$ and 9-fold in $(k_{cat.}/K_m)_D$.

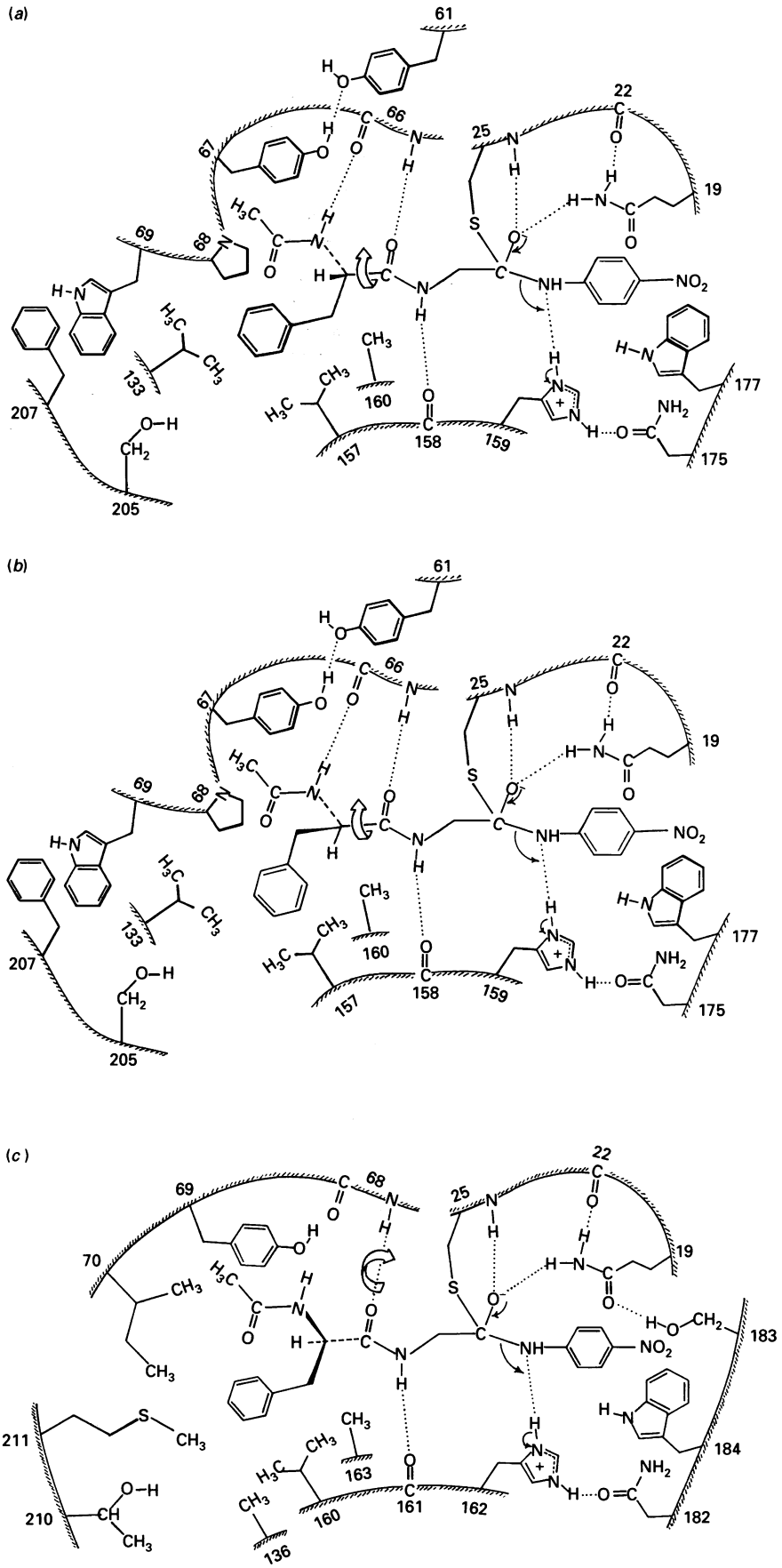
In the case of papain-catalysed hydrolysis of substrates (I) and (II) the results of our earlier model building (Kowlessur *et al.*, 1990, and see also Templeton *et al.*, 1990) demonstrated the possibility of making $P_2$–$S_2$ and catalytic-site contacts and also the three hydrogen bonds shown in Scheme 1 for the tetrahedral intermediates (THIs) derived from both of the enantiomeric substrates. In the THI from the papain–L-enantiomer combination, the $(P_2)\alpha$-H atom projects out of the protein and the $\alpha$-benzyl side chain is accommodated such that $C_{(\beta)}$ lies between the

**Table 1. Values of the specificity constant and of the index of stereochemical selectivity for the hydrolysis of the enantiomeric *N*-acetyl-Phe-Gly 4-nitroanilides catalysed by papain, ficin and actinidin at 25 °C in 8.3% (v/v) dimethylformamide at pH 6.0**

The results for the specificity constant are best-fit values ± standard errors.

| | Number of $v$, [S] data pairs | | Specificity constant, $k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$) | | | |
|---|---|---|---|---|---|---|
| | L-Enantiomer | D-Enantiomer | L-Enantiomer | D-Enantiomer | $I_{ss}$ | Relative values of parameters for papain:ficin:actinidin |
| Papain | 32 | 15 | 2064* ± 31 | 5.5* ± 0.1 | 375 | |
| Ficin | 21 | 32 | 271 ± 6 | 2.9 ± 0.1 | 93 | |
| Actinidin | 26 | 18 | 13.3 ± 0.7 | 0.34 ± 0.01 | 39 | |
| $(k_{cat.}/K_m)_L$ | | | | | | 155:20:[1] |
| $(k_{cat.}/K_m)_D$ | | | | | | 16:8.5:[1] |
| $I_{ss}$ | | | | | | 9.6:2.4:[1] |

* These values result from analysis of the combination of new data [$(k_{cat.}/K_m)_L$ = 2094 ± 38 $M^{-1}\cdot s^{-1}$ and $(k_{cat.}/K_m)_D$ = 5.4 ± 0.01 $M^{-1}\cdot s^{-1}$ (G. W. Mellor, E. W. Thomas, D. Kowlessur, W. Templeton & K. Brocklehurst, unpublished results) with data [$(k_{cat.}/K_m)_L$ = 2040 ± 48 $M^{-1}\cdot s^{-1}$ and $(k_{cat.}/K_m)_D$ = 5.9 ± 0.07 $M^{-1}\cdot s^{-1}$] reported previously (Kowlessur *et al.*, 1990).




**Scheme 2.** For legend see facing page.

side chains of Pro-68 and Ala-60, and beyond it the phenyl ring is adjacent to the side chains of Val-133 and Val-157, which with Try-67, Trp-69 and Phe-207 form the $S_2$ hydrophobic binding pocket. This binding mode is analogous to that deduced by Drenth *et al.* (1976) by X-ray crystallographic studies on papain–inhibitor combinations such as papain alkylated on $S_{(\gamma)}$ of Cys-25 by reaction with the terminal methylene group of *N*-benzyloxycarbonyl-L-Phe-L-Ala-$CH_2Cl$, probably via a hemithioketal intermediate (Brocklehurst & Malthouse, 1978). In the THI from the papain–D-enantiomer combination it appears to be possible for all three of the hydrogen bonds shown in Scheme 1 to be maintained, and even though the $(P_2)\alpha$-H atom projects into the protein the $\alpha$-benzyl side chain can still be accommodated either close to the region in the $S_2$ subsite occupied by this side chain in the complex with the L-enantiomer or by rotation about $C_{(\alpha)}$–$C_{(\beta)}$, with the phenyl ring close to Tyr-67. In the present work, additional model building has suggested ways in which the most obvious binding modes may differ from intermediate to intermediate as the papain-catalysed reaction proceeds through adsorptive enzyme–substrate complex (ES) to tetrahedral intermediate (THI) and thence to acylenzyme (thiol ester) intermediate (ES′) (eqn. 1):

$$E + S \rightleftharpoons ES \rightleftharpoons THI \rightarrow ES'(+P_1) \rightarrow \quad (1)$$

The negative Hammett rho value found for the dependence of the acylation rate constant on leaving-group structure in substituted *N*-acetyl-L-Phe-Gly anilides (Lowe & Yuthavong, 1971*b*) is consistent with rate-determining general acid-catalysed elimination of the leaving group from an anionic tetrahedral intermediate to produce the acylenzyme, as illustrated in Scheme 2. Model building suggests that in ES (not shown) the three hydrogen-bonding interactions indicated in Scheme 1 can be at least close to the optimal conformation with linear > C=O ... H–N < arrangements if it is assumed that the additional hydrogen bond in the catalytic site involving (His-159)–$Im^+H$ and the leaving group is not made at this stage of the catalysis. When it is necessary for this hydrogen bond to be made at the transition-state stage of catalysis to provide for the general acid-catalysed expulsion of the leaving group, an additional constraint is imposed on the binding mode of the substrate moiety. Satisfactory alignments at the catalytic site involving the leaving group and the oxyanion developed from the carbonyl oxygen atom seem to be capable of being achieved only if the $(P_1)$ C–NH–CO–C $(P_2)$ dihedral angle could be approx. 135°. Since this dihedral angle is constrained by partial double-bond character in the C–N bond to be approx. 180°, simultaneous optimization of all of the hydrogen-bonding interactions together with the $P_2$–$S_2$ contacts appears to be impossible. Alignment in the catalytic site can be improved, however, by a small rotation of the substrate moiety about its long axis. Although this rotation might weaken the trans-cleft hydrogen bonds somewhat, it appears to have its greatest effect in the $S_2$ subsite, whereby the $\alpha$-benzyl side chain of the Phe residue moves relative to the hydrophobic side chains of the binding pocket. For the L-enantiomer, the side chain moves within the pocket, bringing $C_{(\beta)}$ closer to the side chains of Val-133 and Val-157. The combination of this readjustment within the $S_2$ subsite and the additional hydrogen bond to the leaving group could provide for more effective binding in the transition state that in the adsorptive complex. For the D-enantiomer, however, $C_{(\beta)}$ moves further into the solvent and the aromatic ring is brought closer to the protein–solvent interface and the contribution of interaction within the $S_2$ subsite to transition state stabilization will be less in this case. The greater the extent of this type of rotational adjustment needed in related enzyme–substrate systems, the greater would be the difference in reactivity predicted between L- and D-enantiomeric substrates. Inspection of the molecular model does not reveal any obvious constraints to progression from the ES binding mode to the THI binding mode discussed above. It must remain a possibility, however, that the binding mode perceived for ES is not an obligatory precursor of that perceived for the THI on the catalytic pathway and it may indeed be a non-productive mode.

Hanzlik *et al.* (1991) reported the unpublished findings of J. Zygmunt that the value of $k_{cat.}/K_m$ for the papain-catalysed hydrolysis of *N*-acetyl-D-Phe-Gly-ONp is smaller than that for the L-enantiomer by a factor of only 8 and interpreted this in general terms as an example of the 'reactivity–selectivity principle.' This principle relates to the situation encountered frequently in non-enzymic chemistry in which highly reactive chemical species tend to be non-discriminating and react randomly while less reactive species are more highly selective. The phenomenon may be accounted for in terms of early and late transition states by application of the well-known Hammond postulate. For reactions involving enzymes, it will often be necessary to give detailed consideration to the variety of binding interactions and their interplay with each other and with the covalency changes involved in catalytic-site chemistry if an interpretation in molecular terms is to be achieved. The Zygmunt data might reflect the lack of need to provide for general acid-catalysed assistance to the departure of the good 4-nitrophenolate leaving group from these substrates where THI formation appears to be rate-determining (see Lowe & Yuthavong, 1971*b*). Relaxation of the requirement for a (His-159)–$Im^+H$ ... leaving-group hydrogen bond would diminish the extent of the need for the rotational adjustment discussed above and thus provide for lower stereochemical selectivity.

In the case of actinidin-catalysed hydrolysis of substrates (I) and (II), the value of $(k_{cat.}/K_m)_L$ is decreased by a factor of 155 from the value for the papain reaction (from 2064 $M^{-1} \cdot s^{-1}$ to

**Scheme. 2. Diagram showing postulated binding modes for tetrahedral intermediates (THIs) and transition states proposed for the acylation of the thiol group (*a*) and (*b*) of papain by *N*-acetyl-L-Phe-Gly 4-nitroanilide and its D-enantiomer respectively and (*c*) of actinidin by the D-enantiomer**

Each drawing shows a view looking into the active-centre cleft towards the centre 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 that relative dispositions around the cleft and their approximate spatial relationships to the ligand. Without the small curly arrows that indicate changes of electron density required for the general acid-catalysed expulsion of the 4-nitroaniline leaving group, the drawings represent THIs; with the curly arrows they represent transition states. The large ribbon arrows indicate the sense in which the substrate moiety needed to be rotated from its perceived position in the adsorptive ES complex in order to make the hydrogen bond from the catalytic site imidazolium cation to the leaving group with the retention of the trans-cleft hydrogen bonds shown. For (*a*) and (*b*) the rotation is about the long axis of the substrate moiety. For (*a*) (papain and the L-enantiomer), the $\alpha$-benzyl side chain of the Phe residue moves within the pocket bringing $C_{(\beta)}$ closer to the side chains of Val-133 and Val-157. For (*b*) (papain and the D-enantiomer), $C_{(\beta)}$ moves further into the solvent and the aromatic ring is brought closer to the protein–solvent interface. For (*c*) (actinidin and the D-enantiomer), and for actinidin and the L-enantiomer (not shown) the rotation is about the axis of the (Gly-68) > NH ... O=C < $(P_2)$–$(P_1)$ > N–H ... O=C < (Asp-161) bridge. A necessary consequence of this for both enantiomers is the sacrifice of the $(P_2)$ > N–H ... O–C < (Gly-68) hydrogen bond. For further details see the text.

13.3 $M^{-1} \cdot s^{-1}$) whereas the value of $(k_{cat.}/K_m)_D$ is decreased by a factor of only 16 (Table 1). These effects combine to produce a substantially smaller value of $I_{ss}$ for the actinidin-catalysed reactions (39) than the analogous value for papain (375). Model building for the actinidin–substrate combinations suggests that, even at the ES complex stage of catalysis, it is not possible to optimize the $P_2$–$S_2$ contacts and the three hydrogen-bonding interactions analogous to those shown in Scheme 1, in the absence of significant movement in protein conformation.

The $S_2$ subsite in actinidin is smaller than that in papain, being confined to a region of the active-centre cleft lined by the hydrophobic side chains of Ile-70, Ala-163, Ala-136, Tyr-69, Val-160 and Met-211. The last of these lies across the cleft and delimits the site. In the corresponding binding area of papain, the hydrophobic region is much larger, involving Tyr-61, Tyr-67, Pro-68, Trp-69, Phe-207 and Ala-160 in the main cleft and extending into the R-domain of the enzyme in a pocket lined by Val-133, Val-157, Ser-205 and Thr-204. One consequence of these differences is that, for substrates with a large hydrophobic side chain at $P_2$, binding to papain will always be better than binding to actinidin when comparing analogous situations, i.e. D with D, L with L, ES with ES and THI (and transition state) with THI (and transition state).

In the actinidin–L-enantiomer adsorptive complex, hydrogen bonds analogous to those shown in Scheme 1, i.e. $(P_1) > N\text{-}H \ldots O{=}C < (Asp\text{-}161)$, $(P_2) > N\text{-}H \ldots O{=}C > (Gly\text{-}68)$, and $(P_2) > C{=}O \ldots H\text{-}N < (Gly\text{-}68)$, can be made provided that steric alignment of the scissile bond with catalytic-site residues is not sought at this stage. It is then possible for the $(P_2)$ Phe side chain to be accommodated in the $S_2$ subsite, the aromatic ring making hydrophobic contacts with Ile-70, Met-211, Ala-136 and Ala-163, while the *N*-acetyl group lies close to Tyr-69. Without the requirement for hydrogen-bonding interactions with the catalytic site at this stage, the 4-nitrophenyl group of the substrate can lie comfortably over His-162 and in hydrophobic contact with Trp-184. At the THI and transition state stages of catalysis it would seem to be necessary to provide for the following additional requirements: (i) to bring the $(P_1)$-carbonyl carbon atom within bonding distance with the (Cys-25)-sulphur atom, (ii) to align the 4-nitroaniline leaving group with (His-162)–$Im^+H$ for hydrogen-bonding and hydron transfer, and possibly (iii) to position the $(P_1)$-carbonyl oxygen atom for stabilization of the oxyanion of the THI by hydrogen-bonding with (Cys-25) > N–H and (Gln-19)–$NH_2$. None of these additional requirements appear capable of being met without disturbing the trans-cleft hydrogen-bonding interactions described above for the ES complex. If, however, the substrate molecule is rotated about the axis of the $(Gly\text{-}68) > N\text{-}H \ldots O{=}C < (P_2)\text{-}(P_1) > N\text{-}H \ldots O{=}C < (Asp\text{-}161)$ bridge, the scissile bond and its attached groups are brought down into the vicinity of the catalytic site, and all of the requirements described in (i)–(iii) can be met. Necessary consequences of these adjustments are that the $(P_2) > N\text{-}H \ldots O{=}C < (Gly\text{-}68)$ hydrogen bond must be sacrificed, and $(P_2)$-$C_\alpha$ moves closer to the protein–solvent interface. Removal of the conformational constraint imposed by the $(P_2) > N\text{-}H \ldots O{=}C < (Gly\text{-}68)$ hydrogen bond allows rotation about the $(P_2)C_\alpha$–C=O axis. This, together with the other rotations about $C_\alpha$–$C_\beta$ and $C_\beta$–phenyl, permits the Phe side chain to take up a position in the $S_2$ subsite that appears to be only slightly less favourable than that in the adsorptive complex. The *N*-acetyl group, however, is obliged to move out of the shelter of Tyr-69 and into the solvent. It is important to note that the need for rotation to achieve the required binding interactions in the THI of the actinidin reaction is more pronounced than in the THI of the papain reaction and that the nature of rotation is different in the two cases. In the case of papain, the larger $S_2$ subsite allows the catalytic-site interactions to be achieved by rotation around the long axis of the substrate without breaking the $(P_2) > N\text{-}H \ldots O{=}C < (Gly\text{-}66)$ hydrogen bond.

In the actinidin–D-enantiomer adsorptive complex, the three *trans*-cleft hydrogen bonds analogous to those shown in Scheme 1 can be made, with the leaving group occupying a hydrophobic site adjacent to Trp-184, as in the actinidin–L-enantiomer complex. Hydrophobic contacts with Tyr-69, Ala-136 and Val-160 can be maintained but those with Ile-70, Met-211 and Ala-163, which can be made in the complex with the L-enantiomer, are lost. Binding in the ES complex appears to be necessarily much poorer for the D-enantiomer than for the L-enantiomer. As with the L-enantiomer, formation of the necessary catalytic-site contacts in the THI derived from the the D-enantiomer can be achieved by rotation of the substrate about the axis of the trans-cleft hydrogen-bonding bridge. As before, the $(P_2) > N\text{-}H \ldots O{=}C < (Gly\text{-}68)$ hydrogen bond must be sacrificed and $(P_2)$-$C_\alpha$ moves further towards solvent. The major differences in the binding modes of the THIs pertaining to the reactions of papain with the L- and D-enantiomers (Pap–L and Pap–D) and of actinidin with the D-enantiomer (Act–D) postulated above are illustrated in Scheme 2(*a*–*c*).

In order to account for the order of reactivity reflected in $k_{cat.}/K_m$, i.e. Pap–L (2064 $M^{-1} \cdot s^{-1}$) $\gg$ Act–L (13 $M^{-1} \cdot s^{-1}$) > Pap–D (5.5 $M^{-1} \cdot s^{-1}$) $\gg$ Act–D (0.34 $M^{-1} \cdot s^{-1}$), it is necessary to consider the effectiveness of the binding interactions in the transition states for the four reactions, which should be approximated by the effectivness of binding in the THIs. In comparing Pap–L–THI with Pap–D–THI, it is clear why binding in the latter is worse than in the former. The rotation required about the long axis of the substrate structure moves the $(P_2)$-Phe side chain further into the $S_2$ binding pocket for Pap–L–THI but further towards solvent for the Pap–D–THI. Comparison of Pap–D–THI with Act–L–THI is more difficult because the nature of the rotation necessary to make the catalytic-site contacts in the actinidin systems is different from that in the papain systems. However, although binding in Act–L–THI appears to demand the loss of the $(P_2) > N\text{-}H \ldots O{=}C < (Gly\text{-}68)$ hydrogen bond, movement of the $(P_2)$-Phe side chain towards solvent is significantly less than for Pap–D–THI and this may account for the relative similarity in the values of $k_{cat.}/K_m$ for the Pap–D and Act–L reactions (5 $M^{-1} \cdot s^{-1}$ and 13 $M^{-1} \cdot s^{-1}$ respectively). The less effective binding of the $(P_2)$-Phe side chain in Act–D–THI than in Act–L–THI described above could account for the relative values of $k_{cat.}/K_m$ for the reactions proceeding through these THIs (0.34 $M^{-1} \cdot s^{-1}$ and 13 $M^{-1} \cdot s^{-1}$ respectively). The poor binding of $(P_2)$-Phe in the Act–D–THI together with the loss of the $(P_2) > N\text{-}H \ldots O{=}C < (Gly\text{-}68)$ hydrogen bond presumably accounts for the lower value of $k_{cat.}/K_m$ for the Act–D reaction (0.34 $M^{-1} \cdot s^{-1}$) than for the Pap–D reaction (5.5 $M^{-1} \cdot s^{-1}$).

For the ficin-catalysed hydrolysis of substrates (I) and (II) the value of $I_{ss}$ (93) lies between those of papain (375) and actinidin (39). The value relates to that for the papain reactions as arising from a decrease in $(k_{cat.}/K_m)_L$ by a factor of 7.6 and a decrease in $(k_{cat.}/K_m)_D$ by a factor of 1.9. The intermediate position occupied by ficin in the papain, ficin, actinidin series suggests that a three-dimensional crystal structure of ficin, and structure-related kinetic studies on reactions of this enzyme, may contribute valuable additions to the developing picture of structure–function relationships in the cysteine proteinase family.

## Concluding comments

Qualitatively the stereochemical selectivity ($I_{ss}$) for the *N*-acetyl-Phe-Gly 4-nitroanilides decreases in the same order, papain > ficin > actinidin, as the kinetic specificity ($k_{cat.}/K_m$).

Quantitatively the relationships are more complex, reflecting differential effects on $(k_{cat.}/K_m)_L$ and $(k_{cat.}/K_m)_D$ with change in enzyme structure. The large decreases in $(k_{cat.}/K_m)_L$ in the series papain > ficin > actinidin are not paralleled closely by the decrease in $(k_{cat.}/K_m)_D$, with only a small difference in this parameter between papain and ficin and a large difference between ficin and actinidin. The substrate-catalysis data presented for these three analogous enzymes seem to suggest that the more effectively an enzyme is able to utilize a combination of binding interactions in promoting catalysis, the more effectively will it discriminate between a pair of substrates in which a particular binding interaction is significantly better for one member of the pair than for the other. Where the kinetic specificity is lower, there may be more opportunities to establish satisfactory, though suboptimal, binding modes for both the L-and D-enantiomer in their associated transition states.

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