Comparative resonance Raman spectroscopic and kinetic studies of acyl-enzymes involving papain, actinidin and papaya peptidase II

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Resonance Raman spectra are reported for a series of dithioacyl-enzymes involving actinidin (EC 3.4.22.14) and papaya peptidase II (the more basic monothiol cysteine proteinase of *Carica papaya*). The acyl groups are *N*-benzoylglycine and *N*-(β -phenylpropionyl)glycine containing C=S or $^{13}\text{C}=\text{S}$ at the ester function. Comparison of the data with those for the corresponding papain (EC 3.4.22.2) analogues [Storer, Lee & Carey (1983) *Biochemistry* 22, 4789–4796] allows us to define the conformation of the dithioacyl group in the catalytic site. In each case the dithioacyl group is bound in a single conformation known as conformer B, in which the glycinic nitrogen atom comes into close contact with the sulphur atom of the catalytic-site cysteine residue. For the *N*-(β -phenylpropionyl)glycine dithioacyl-enzymes the torsional angles of the $\text{NH}-\text{CH}_2-\text{C}(=\text{S})$ bonds assume values typical of an essentially relaxed non-strained state. However, for the *N*-benzoylglycine dithioacyl-enzymes there is evidence for a slightly perturbed conformer B, and the perturbation is most pronounced for *N*-benzoylglycine dithioacyl-actinidin. Values of k_{+2}/K_s and k_{+3} for the reactions of papain, actinidin and papaya peptidase II with *N*-benzoylglycine and *N*-(β -phenylpropionyl)glycine methyl thionoesters were obtained by a pre-steady-state kinetic study. Wide variation was found in k_{+2}/K_s , but the values of k_{+3} are all similar. This general picture is supported by the results from a steady-state kinetic study of the reactions of the three enzymes with *N*-benzoyl-L-arginine-*p*-nitroanilide and with *N*-benzyloxycarbonyl-L-lysine-*p*-nitrophenyl ester. The similarity of the values of k_{+3} , together with the invariance of conformer B geometry at the P_1 site, suggests that the chemistry of the deacylation process is highly conserved among these three cysteine proteinases.

The cysteine proteinases (for reviews see Lowe, 1976; Brocklehurst *et al.*, 1981a; Polgár & Halász, 1982) are a group of enzymes in which a catalytic-site thiol group is transiently acylated during the catalytic act. Lowe & Williams (1965) showed that thionoester substrates $[\text{R}-\text{C}(=\text{S})-\text{OR}']$ react with the thiol group of the cysteine-25 residue of papain to produce dithioacyl-enzymes $[\text{R}-\text{C}(=\text{S})-\text{S}-\text{Enzyme}]$

Abbreviations used: Bz-Arg-Nan, *N*-benzoyl-L-arginine-*p*-nitroanilide; Z-Lys-ONp, *N*-benzyloxycarbonyl-L-lysine-*p*-nitrophenyl ester; Nbs $_2^{2-}$, 5,5'-dithiobis-(2-nitrobenzoic acid); PPII, papaya peptidase II; r.R., resonance Raman.

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as intermediates in the catalytic process. The dithioester intermediate differs from the natural thioester by the substitution of C=S for the C=O group. The $k_{\text{cat.}}/K_m$ values are similar for oxygen and thionoester substrates although the $k_{\text{cat.}}$ values for the latter are 20–30-fold less than those for the oxygen analogues (Lowe & Williams, 1965). Kinetic studies reveal that, in all likelihood, the mechanisms are the same for the two classes of substrate (A. C. Storer & P. R. Carey, unpublished work). The use of an excess of substrate gives rise to a quasi-steady-state population of intermediate. The dithioester group in the catalytic site has a λ_{max} near 315 nm, and irradiation of the reaction mixture with 324 nm Kr^+ laser light generates the

r.R. spectrum of the dithioester moiety (Storer *et al.*, 1979). Interpretation of the r.R. spectra for *N*-acylglycine dithioacyl-papains shows that there is a single homogeneous conformational population of bonded acyl groups, designated conformer B (Carey & Storer, 1983; Storer *et al.*, 1983). The torsional angle about the C–C bond in the glycine $\text{NH}-\text{CH}_2-\text{C}(=\text{S})-\text{S}$ moiety assumes a value such that the amide N atom comes into close contact with the thiol S atom. The conformation about the glycine bonds is essentially relaxed (Lee *et al.*, 1983; Storer *et al.*, 1983). For *N*-benzoylglycine substrates there is evidence for, at most, only a small distortion of the glycine angles away from those of the corresponding ethyl dithioester in its relaxed state in solution. Furthermore, no evidence has been found for major distortion in the $-\text{C}(=\text{O})\text{NH}$ -peptide group of the bonded acyl group. However, there is evidence for a non-relaxed conformation in the cysteine $-\text{S}-\text{C}_\beta-\text{C}_\alpha$ heavy-atom skeleton for the native dithioacyl-papain with the C_α atom being significantly out of the plane defined by the $\text{C}(=\text{S})-\text{S}-\text{C}$ atoms (K. I. Varughese, A. C. Storer & P. R. Carey, unpublished work).

In the present work, actinidin and papaya peptidase II were selected for comparison with papain. Papaya peptidase II (PPII) (originally called papaya peptidase A) is the other monothiol cysteine proteinase present in papaya latex (Brocklehurst & Salih, 1983). By using two-protonic-state reactivity probes (see Brocklehurst, 1982) all three of these enzymes have been shown to possess interactive catalytic-site systems (possibly containing $-\text{S}^--\text{ImH}^+$ ion-pairs) that maintain the thiol group in a nucleophilic and catalytically competent state in acidic media. However, the characteristics of these nucleophilic interactive systems, which must derive from their micro-environments possibly controlled by interactions remote from the immediate catalytic-site region, are considerably different from each other (Salih & Brocklehurst, 1983, and references cited therein). The marked differences in catalytic-site properties between papain and actinidin are particularly noteworthy in view of the close similarities in structure suggested by the X-ray-diffraction studies (Drenth *et al.*, 1976; Baker, 1980). This contrast is further highlighted by the work reported in the present paper. The r.R. and kinetic data lead to the conclusion that the chemistry of the deacylation process is highly conserved among the cysteine proteinases papain, actinidin and PPII. Thus the structural differences between these enzymes detected by differences both in thiol-group reactivity and in the kinetics of the overall acylation process do not play a major part in the chemistry of the deacylation step.

Materials and methods

Bz-Arg-Nan and Z-Lys-ONp were obtained from Sigma Chemical Co. Unlabelled and $^{13}\text{C}=\text{S}$ -substituted *N*-benzoylglycine and *N*-(β -phenyl-propionyl)glycine methyl thionoesters were prepared and characterized as described by Ozaki *et al.* (1982) and by Storer *et al.* (1983).

The enzymes were prepared as described previously: for papain by Storer *et al.* (1983) or by Baines & Brocklehurst (1979), for actinidin by Brocklehurst *et al.* (1981b) and for PPII (papaya peptidase A) by Baines & Brocklehurst (1982).

R.R. spectra were obtained by using 324 nm Kr^+ excitation from a Coherent Radiation 3000K laser. The sample cell consisted of a 1 cm-square quartz cuvette, stirred by a small magnetic 'pip'. The vertical laser beam entered the bottom of the cell and the scattered light was observed at 90° . The scattered light was collected by a Cassegrain optic and analysed with a Spex Triplemate spectrometer. The photons at the exit port of the Triplemate were detected by a u.v.-sensitive Tracor Northern TN-6132 multichannel diode array. The instrumentation is described in detail in Carey & Sans Cartier (1983).

The reaction mixture used to obtain r.R. spectra typically had a total volume of 0.7 ml and contained $150\ \mu\text{M}$ enzyme, 8 mM substrate, 10–20% acetonitrile and sodium phosphate buffer at pH 6.0. The r.R. spectra in Figs. 1–4 contain an intense peak near 920 cm^{-1} and a weak feature near 750 cm^{-1} due to acetonitrile. A band is also observed near 980 cm^{-1} due to SO_4^{2-} . Each enzyme is stored in concentrated $(\text{NH}_4)_2\text{SO}_4$ solution and activated by dilution with the phosphate buffer, pH 6.0, and adding 2-mercaptoethanol ($3\ \mu\text{l}/\text{ml}$ of enzyme). The enzyme is then passed through a Sephadex G-15 column ($14\text{ cm} \times 1\text{ cm}$) to remove the 2-mercaptoethanol before use in the reaction mixture. From the r.R. spectra (Figs. 1–4) it is apparent that some of the SO_4^{2-} ions migrate with the protein on the column.

Pre-steady-state kinetic studies on the thionoester substrates were carried out at 315 nm with a stopped-flow spectrometer (Cantech Scientific, Winnipeg, Manitoba, Canada) coupled via a transient recorder to a Commodore Pet microcomputer. The data for the time-dependent increase in A_{315} (256 points, 8-bit resolution) were fitted to a single exponential function by using a program based on Marquardt's non-linear least-squares fitting procedure (Marquardt, 1963). Measurements were made at 20°C at pH 6.5 in a buffer containing $50\text{ mM}-\text{Na}_2\text{HPO}_4$, $0.2\text{ M}-\text{NaCl}$, $5\text{ mM}-\text{EDTA}$ and 20% (v/v) acetonitrile (papain) or 10% (v/v) acetonitrile (actinidin and papaya peptidase II). The enzyme concentrations used were $5\ \mu\text{M}$,

7 μM and 3 μM for papain, actinidin and PPII respectively. The papain and PPII concentrations were determined by using Nbs_2^{2-} (Ellman, 1959), and the concentration of actinidin was determined by using $\epsilon_{280} = 55120 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Brocklehurst *et al.*, 1979). For *N*-benzoylglycine methyl thionoester the substrate concentration ranges, number of concentrations and number of replicates were 0.4–6.0 mM, 6 and 2–5, 2.0–5.0 mM, 4 and 3–6, and 0.2–5.0 mM, 7 and 3–4 for papain, actinidin and PPII respectively. The corresponding respective values for *N*-(β -phenylpropionyl)glycine methyl thionoester were 0.05–2.0 mM, 6 and 3–5, 0.2–3.0 mM, 6 and 3–4, and 0.2–3.0 mM, 6 and 3–6.

Steady-state kinetic studies with Bz-Arg-Nan as substrate were carried out at 25°C, 10.1 and pH 6.5 and 7.5 with a Cary 118C spectrophotometer. Initial rates of release of 4-nitroaniline were obtained from measurements at 410 nm and $\Delta\epsilon_{410} = 8800 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Erlanger *et al.*, 1961). Reactions were carried out at the following active enzyme concentrations: papain, 1.3 μM , actinidin, 1.0 μM , and PPII, 2–3 μM . The ranges of substrate concentration used (for the papain reactions 0.05–0.35 mM, for actinidin 0.05–0.25 mM and for PPII 0.05–6.0 mM) were such that plots of v versus $[S]$ were linear, i.e. $[S] \ll K_m$. Values of $k_{\text{cat.}}/K_m$ were calculated from $k_{\text{cat.}}/K_m = v/[E][S]$, where $[E]$ is the enzyme active-site concentration, measured by titration with 2,2'-dipyridyl disulphide at pH 4 (see Brocklehurst, 1982).

Steady-state kinetic studies with Z-Lys-ONp were carried out at 25°C, 10.1 and pH 5.5 with a Dionex stopped-flow system. Chromophore release was monitored at 320 nm and quantified by using $\epsilon_{320} = 7920 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Complete progress curves for substrate hydrolysis were recorded with the use of the following concentrations: for papain, $[E] = 0.03 \mu\text{M}$, $[S] = 2\text{--}5 \mu\text{M}$; for actinidin, $[E] = 1.37\text{--}1.9 \mu\text{M}$, $[S] = 15\text{--}20 \mu\text{M}$; for PPII, $[E] = 1 \mu\text{M}$, $[S] = 30\text{--}45 \mu\text{M}$. The progress curves were analysed by the half-time method of Wharton & Szawelski (1982) to provide values of K_m and V , the latter being used with the value of $[E]$ to provide $k_{\text{cat.}}$. Plots of $t_{1/2}$ against arbitrarily chosen $[S]_0$ were linear, with slope $1/2V$ and intercept $\ln 2 \cdot K_m/V$. It should be noted that in the paper by Wharton & Szawelski (1982, p. 353, column 1, line 9) the value of the slope is printed incorrectly as $1/2V$.

Results and discussion

R.R. spectra of dithioacyl-actinidin and dithioacyl-PPII

The r.R. spectra of *N*-benzoylglycine and *N*-(β -phenylpropionyl)glycine dithioacyl-PPII are shown in Figs. 1 and 2 respectively. The corresponding r.R. spectra for dithioacyl-actinidin are

shown in Figs. 3 and 4. Each Figure also contains the r.R. spectrum of the $^{13}\text{C}=\text{S}$ -substituted analogue.

The r.R. spectrum of *N*-benzoylglycine dithioacyl-PPII in Fig. 1 is identical with that of the papain analogue reported in Storer *et al.* (1983). Thus, by analogy to the papain case, we may assign the 1172 cm^{-1} peak to a coupled mode involving the $\text{C}(=\text{O})\text{--NH--CH}_2\text{--C}(=\text{S})\text{--S}$ moiety, the intense 1129 cm^{-1} peak to $\nu_{\text{C}=\text{S}} + \nu_{\text{C--C}}$ [from the $\text{CH}_2\text{--C}(=\text{S})$], the weak 674 cm^{-1} feature to $\nu_{\text{S--C}}$, and the band at 596 cm^{-1} to a highly coupled mode involving C–C, C=S and C–S stretching motions. The only substantive spectral change upon $^{13}\text{C}=\text{S}$ substitution is the shift of the 1129 cm^{-1} feature to 1094 cm^{-1} , and this again strongly resembles the papain finding.

The r.R. spectrum of *N*-(β -phenylpropionyl)glycine thioacyl-PPII (Fig. 2) is also identical with that of the papain analogue, and the peak assignments correspond closely to those made for the *N*-benzoylglycine substrate. There is, however, an additional r.R. peak for the β -phenylpropionyl derivative occurring at 1094 cm^{-1} in both Fig. 2 and the analogous thioacyl-papain spectrum (Storer *et al.*, 1983). This is the so-called band III (Ozaki *et al.*, 1982), and consists of $\nu_{\text{C=S}} + \nu_{\text{C--C}}$ + components from other bonds in the glycine moiety. The substitution of $^{13}\text{C}=\text{S}$ brings about complex changes in the 1100 cm^{-1} region, and these again mimic the papain findings.

Confirmation that the r.R. peaks do indeed correspond to the dithioacyl-enzyme intermediate is given in the spectra in Figs. 1 and 2 taken some time after the addition of substrate to the enzyme. The bottom traces, taken 30 min (Fig. 1) and 8 min (Fig. 2) after starting the reaction, when the substrate supply is exhausted, contain no trace of the peaks assigned to the intermediate.

The very close parallel between the papain and PPII r.R. results for the two substrates discussed here allows us to conclude that each acyl group takes up an identical conformation in both active sites. Hence, like papain, the PPII site selects one of the available conformational states available to *N*-acylglycine dithioesters, namely conformer B. The torsional angles in the substrate's $\text{C}(=\text{O})\text{--NH--CH}_2\text{--C}(=\text{S})\text{--S}$ bonds assume essentially relaxed unstrained values in the active site, although, as in the case of papain, there is a slight perturbation away from the unstrained conformer for the *N*-benzoylglycine substrate. No evidence is found for the presence of a second conformer in the active site of PPII.

The situation for actinidin is slightly different. The r.R. spectra (Figs. 3 and 4) are of poorer quality, reflecting the smaller population of acyl-enzyme (k_{+2}/K_s values are considerably smaller

Fig. 1. R.R. spectra of unlabelled (middle trace) and $^{13}\text{C}=\text{S}$ -substituted (top trace) *N*-benzoylglycine dithioacyl-PPII taken 20 s and 180 s after reaction started respectively

The bottom trace was recorded 30 min after mixing. R.R. conditions: 324 nm excitation, ≈ 100 mW, 12 cm^{-1} spectral slit, spectral acquisition time 20 s.

for actinidin; see Table 1). The r.R. peaks may be assigned in a similar fashion to those for the PPII spectra and the same conclusions drawn concerning conformer B selection. Moreover, within the limits of experimental error the peak positions for the *N*-(β -phenylpropionyl)glycine dithioacyl-actinidin r.R. spectra are the same as those for corresponding papain (Storer *et al.*, 1983) and PPII analogues. However, for *N*-benzoylglycine dithioacyl-actinidin there is a small shift in the position of the intense peak near 1130 cm^{-1} . The position was measured with reference to the aceto-

nitrile feature at 922 cm^{-1} . For six different *N*-benzoylglycine dithioacyl-actinidin preparations the band appeared at $1133 \pm 1\text{ cm}^{-1}$, whereas in the case of the papain derivative it occurs at $1129 \pm 1\text{ cm}^{-1}$. The signal-to-noise ratio prevents us from making such a precise comparison for the less-intense features in the 600 cm^{-1} region.

Thus it can be concluded that, whereas all three dithioacyl-enzymes derived from the *N*-(β -phenylpropionyl)glycine methyl thionoester substrate possess essentially the same $\text{NH}-\text{CH}_2-\text{C}(=\text{S})$ torsional angles as does the 'relaxed' *N*-(β -phenyl-

Fig. 2. R.R. spectra of unlabelled (middle trace) and $^{13}\text{C}=\text{S}$ -substituted (top trace) *N*-(β -phenylpropionyl)glycine dithioacyl-PPII

The bottom trace was recorded 8 min after mixing, R.R. conditions: 324 nm excitation, $\approx 100\text{ mW}$, 12 cm^{-1} spectral slit, spectral acquisition time 20–50 s.

propionyl)glycine ethyl dithioester in solution, some variation exists in these angles for the *N*-benzoylglycine derivatives. This variation is reflected in the positions of band II for the dithioacyl-enzymes and the model ethyl dithioester, i.e. the band II positions are for *N*-benzoylglycine dithioacyl-actinidin 1133 cm^{-1} , dithioacyl-papain 1129 cm^{-1} , dithioacyl-PPII 1129 cm^{-1} , *N*-benzoyl-ethyl dithioester in solution (H_2O) 1124 cm^{-1} , and in the crystalline state 1118 cm^{-1} . Therefore the torsional angles of the $\text{NH}-\text{CH}_2-\text{C}(=\text{S})$ fragment of *N*-benzoylglycine dithioacyl-actinidin deviate

more from the 'relaxed' state of the model ethyl ester in solution than do the same angles in the papain and PPII derivatives. These deviations are probably quite small, and the consequences of the change in geometry on the strength of the $\text{N}\cdots\text{S}(\text{thiol})$ contact in the active sites and on the deacylation rate constants are discussed in the final section.

Kinetic studies

The minimal kinetic model for cysteine protein-

Fig. 3. R.R. spectra of unlabelled (bottom trace) and ^{13}C =S-substituted (top trace) *N*-benzoylglycine dithioacyl-actinidin, taken approx. 60 s after starting reaction

R.R. conditions: 324 nm excitation, ≈ 100 mW, 12 cm^{-1} spectral slit, spectral acquisition time 30 s.

ase-catalysed hydrolysis is the well-known three-step acyl-enzyme model (eqn. 1) (see, e.g., Polgár & Halász, 1982):

Both acylation (rate constant k_{+2}) and deacylation (rate constant k_{+3}) have been suggested to involve tetrahedral intermediates, and the possibility of kinetically significant conformational transitions cannot be ruled out.

When using the thionoester substrates it is possible to record by stopped-flow spectrophotometry

the rapid pre-steady-state formation of dithioacyl-enzyme as an increase in absorption near to 315 nm. The measured first-order rate constant ($k_{\text{meas.}}$) for this process is related to the parameters appearing in eqn. (1) by eqn. (2) (see Hollaway *et al.*, 1971):

$$k_{\text{meas.}} = \frac{k_{+2}[\text{S}]}{K_s + [\text{S}]} + k_{+3} \quad (2)$$

For the reactions of both thionoester substrates with PPII (Fig. 5) and with actinidin (Fig. 6), plots of $k_{\text{meas.}}$ against $[\text{S}]$ (substrate concentration) were linear over the range of $[\text{S}]$ permitted by substrate solubility. In terms of eqn. (2), the linearity of these

Fig. 4. R.R. spectra of unlabelled (bottom trace) and $^{13}\text{C}=\text{S}$ -substituted (top trace) *N*-(β -phenylpropionyl)glycine dithioacyl-actinidin, taken approx. 20 s after starting reaction

R.R. conditions: 324 nm excitation, ≈ 100 mW, 12 cm^{-1} spectral slit, spectral acquisition time, 15 s.

plots implies that $[\text{S}] \ll K_s$ (where $K_s = k_{-1}/k_{+1}$), and the slope and ordinate intercept (at $[\text{S}] = 0$) provide values of k_{+2}/K_s and k_{+3} respectively. These values are shown in Table 1, together with values of $k_{\text{cat.}}/K_m$ for the enzyme-catalysed hydrolysis of Bz-Arg-Nan and of Z-Lys-ONp, and values of $k_{\text{cat.}}$ for the latter substrate. For the 'slow' anilide substrate values of $k_{\text{cat.}}/K_m$ can be taken as values of k_{+2}/K_s with reasonable confidence, whereas those for the 'fast' reactive ester substrate might not have such simple significance. For this substrate, however, it is probable that deacylation is rate-determining and thus $k_{\text{cat.}}$ approximates closely to k_{+3} .

The main feature of the data in Table 1 is that, although there is considerable variation between the three enzymes in many of the values of k_{+2}/K_s , the values of k_{+3} for all of the dithioacyl-enzymes are remarkably similar to each other. For Z-Lys-ONp also, the values of k_{+3} are essentially invariant with change in the enzyme, although the actual values of k_{+3} are considerably greater than those for the deacylation of the dithioacyl-enzymes. The differences in k_{+2}/K_s (or $k_{\text{cat.}}/K_m$) point to a complex combination of factors that affect the overall acylation process. Thus the orders of reactivity are: for *N*-benzoylglycine methyl thionoester, $\text{PPII} \gg \text{papain} \gg \text{actinidin}$; for

Table 1. Kinetic characteristics of substrate hydrolysis catalysed by papain, actinidin and PPII

The reactions of the two thionoester substrates were carried out at 20°C, *I*0.3 and pH 6.5 in sodium phosphate buffer containing 20% (v/v) acetonitrile for the papain reaction and 10% (v/v) acetonitrile for the other two enzyme reactions. The reactions of Bz-Arg-Nan and Z-Lys-ONp were carried out at 25°C, *I*0.1 and pH 6.5 [sodium phosphate buffer containing 1.5% (v/v) dimethyl sulphoxide] and pH 5.5 [sodium acetate buffer containing 1.6% (v/v) acetonitrile] respectively. The standard errors on the parameters for the reactions of these substrates were generally <10%.

	N-Benzoylglycine methyl thionoester		N-(β-Phenylpropionyl) glycine methyl thionoester		Bz-Arg-Nan	Z-Lys-ONp	
	k_{+2}/K_s (M ⁻¹ ·s ⁻¹)	k_{+3} (s ⁻¹)	k_{+2}/K_s (M ⁻¹ ·s ⁻¹)	k_{+3} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ ·s ⁻¹)	$10^{-7}k_{cat}/K_m$ (M ⁻¹ ·s ⁻¹)	k_{cat} (s ⁻¹)
Papain	0.213 ± 0.007	0.0841 ± 0.002	2.59 ± 0.03	0.165 ± 0.008	204	1.9	30
Actinidin	0.0165 ± 0.003	0.104 ± 0.001	0.198 ± 0.002	0.188 ± 0.003	7.6	0.16	22
PPII	1.01 ± 0.02	0.057 ± 0.03	3.78 ± 0.06	0.11 ± 0.06	21.4	0.18	50

Fig. 5. Plot of the measured exponential rate constants (k_{meas}) against substrate concentration $[S]$ for PPII with the substrates N-(β-phenylpropionyl)glycine methyl thionoester (●) and N-benzoylglycine methyl thionoester (○).

The lines were obtained by a weighted linear-regression procedure, the weights being the reciprocals of the standard deviations calculated from replicates at each substrate concentration.

Fig. 6. Plot of the measured exponential rate constants (k_{meas}) against substrate concentrations $[S]$ for actinidin with the substrates N-(β-phenylpropionyl)glycine methyl thionoester (●) and N-benzoylglycine methyl thionoester (○).

The lines were obtained as described in the legend to Fig. 5.

N-(β-phenylpropionyl)glycine methyl thionoester, PPII ≈ papain > actinidin; for Bz-Arg-Nan, papain > PPII > actinidin; for Z-Lys-ONp, papain > PPII ≈ actinidin.

When compared with this complexity, the relative invariance of related k_{+3} values with change in enzyme is particularly striking.

Substrate conformation, specificity and kinetics

Substrate specificity is most effectively discussed for actinidin and papain, since the X-ray-crystallographically derived structures for these enzymes provide a basis for rationalizing the kinetic data. Baker (1980) compared the structure of papain and actinidin in those parts of the active sites where substrate cleavage occurs, and noted

that the same residues are present in essentially identical orientations. Baker *et al.* (1980) also compared the kinetic parameters k_{cat} , K_m and k_{cat}/K_m for the actinidin-catalysed hydrolyses of N-substituted amino acid esters and amides with the corresponding values for the papain-catalysed reactions. They found that substrates possessing aromatic N-substituents, which would probably bind in the S₂ sub-site [in the Schechter & Berger's (1967) nomenclature], have lower k_{cat}/K_m values for actinidin. This difference was attributed to the absence from actinidin of the extensive hydrophobic binding pocket found in papain at the S₂ sub-site. No trend was observable in their k_{cat} values, which varied less markedly than did k_{cat}/K_m . The results reported by Baker *et al.* (1980)

are in general accord with the proposal by Lowe & Yuthavong (1971) that the specificity of papain and presumably the other cysteine proteinases lies in the acylation step. We see a further support for this idea in Table 1. For both thionoester substrates k_{+2}/K_s is 13-fold less for actinidin than for papain. However, the k_{+3} ($=k_{\text{cat}}$) values do not vary by more than a factor of 2-fold for the same substrate.

The novel aspect of the r.R. results is that we can now introduce a structural aspect for the enzyme-bonded dithioacyl group into the discussion of kinetics and specificity. The insensitivity of the k_{+3} values can be understood in terms of the structural invariance in the enzyme-substrate region that includes the P_1 geometry. The question arises as to why the k_{+3} for *N*-benzoylglycine dithioacyl-actinidin hydrolysis is very similar to that for *N*-benzoylglycine dithioacyl-papain when the r.R. spectra of the intermediates, and therefore their geometries, are measurably different. Part of the answer lies in the fact that the geometric differences are probably quite small and do not give rise to a change in the strength of the $N\cdots S(\text{thiol})$ interaction which is broken in the rate determining step for deacylation (P. R. Carey, H. Lee, Y. Ozaki & A. C. Storer, unpublished work).

The general picture emerging from the r.R. spectra of glycine dithioacyl intermediates of papain, actinidin and PPII is that each enzyme active site selects one of the available conformers and that for any one substrate the conformation is very similar, or the same, for each enzyme. Favourable or unfavourable contacts in the P_2 - S_2 region are not reflected in a marked change of substrate geometry at P_1 when we assess this geometry at the acyl-enzyme state (although these contacts probably have a marked effect during the acylation process). Thus, for substrates based on *N*-acylglycine derivatives, there is no evidence for major substrate strain at the acyl-enzyme stage of the reaction. The r.R. data presented here, taken with the earlier findings for dithioglycine chymopapain, ficin and bromelain derivatives (Carey *et al.*, 1983), show that the B-type geometry is a feature common to the S_1 - P_1 sites of cysteine proteinases. This finding, taken with the striking similarities of the actinidin and papain catalytic sites found by X-ray-crystallographic studies, provide a structural basis for understanding the similarities in k_{+3} values. For each dithioacyl-enzyme, the acyl group assumes the same conformation and is probably surrounded by the same catalytic groups in the same orientations. The spectroscopic, structural and kinetic data taken together show that the

chemistry of the deacylation process is highly conserved among actinidin, papain and PPII.

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