The Specificity of the S₁' Subsite of Papain

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The specificity of the S₁' subsite of the proteolytic enzyme papain was investigated by studying the effect of L- α -amino acid amides on the enzyme-catalysed hydrolysis of *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester and by determining the kinetic parameters for the enzyme-catalysed hydrolysis of some *N*-benzyloxycarbonylglycyl-L-amino acid amides. These studies showed that the S₁' subsite has a predilection for hydrophobic residues, in particular L-leucine and L-tryptophan. The specificity for these residues is manifest in both the binding and acylation steps. *N*-Benzyloxycarbonylglycine amide is not hydrolysed under comparable conditions, indicating that the amide group adjacent to and on the C-terminal side of the peptide bond about to be cleaved makes an important contribution to the rate of the papain-catalysed hydrolysis of peptides.

From a study of the rate and pattern of cleavage of 40 diastereoisomeric peptides of alanine ranging from di- to hexa-peptides, Schechter & Berger (1967) concluded that the active site of papain comprises seven subsites, each capable of accommodating a single amino acid residue of a peptide substrate. Four of these subsites (S₁–S₄) are located on the *N*-terminal side of the point of cleavage of the peptide, and the other three (S₁'–S₃') on the *C*-terminal side, the numbering being away from the site of cleavage. The S₂ subsite was found to be selective for phenylalanine, the cleavage of peptides containing this residue almost always taking place at the next but one peptide bond towards the *C*-terminus (Schechter & Berger, 1968).

The reaction pathway of papain-catalysed hydrolyses is represented minimally by three steps (Lowe, 1970):

where ES is the Michaelis complex, ES' is the acyl-enzyme formed through the thiol group of cysteine-25, P₂ is the *N*-terminal peptide and P₁ is the *C*-terminal peptide of the hydrolysed substrate.

The specificity of papain for peptides containing a phenylalanine residue was shown to be manifest largely in the acylation step, k_2 ; it was also established by kinetic measurements that the enzyme had an important binding capability for the peptide group adjacent to and on the *N*-terminal side of the peptide bond about to be cleaved, and again the specificity was largely manifest in the acylation step (Lowe & Yuthavong, 1971*a*). From these observations a model was proposed for the interaction of substrates

with the S₁ and S₂ subsites which was in general agreement with that proposed from a low-resolution [0.6 nm (6 Å)] X-ray study of papain inhibited with *N*-*t*-butoxycarbonyl-*p*-iodophenylalanyl-leucine (Wolthers *et al.*, 1970).

The S₁' subsite of papain is stereospecific for L-amino acid residues (Berger & Schechter, 1970), but the cleavage pattern of the B chain of oxidized insulin (Johansen & Ottesen, 1968), of glucagon (Desmazeaud, 1972) and of the α and β chains of human haemoglobin (Konigsberg & Hill, 1962; Goldstein *et al.*, 1963) is dominated largely by the S₂ subsite of the enzyme and no reliable conclusions can be drawn as to the specificity of the S₁' site. There is, however, evidence that suggests that the S₁' subsite has a preference for hydrophobic residues. The papain-catalysed hydrolysis of hippurylanilide and hippurylamide show K_s values of 19.4 mM and 200 mM respectively (Lowe & Williams, 1965; Lowe & Yuthavong, 1971*b*), and k_{cat}/K_m for the benzyl esters is about 100 times greater than for the methyl esters of *N*-mesyl- and *N*-acetyl-glycine, although the leaving ability of methanol and benzyl alcohol are similar (Williams *et al.*, 1972*a,b*). Moreover, in the nucleophile-assisted deacylation of *trans*-cinnamoyl-papain, L-tryptophan amide was much more effective than glycine amide although their pK_a values are similar (Brubacher & Bender, 1966). Nucleophiles can also enhance the deacylation rate of acyl-papains derived from more specific substrates, and it has been proposed that these differences in efficiency between nucleophiles that do not correlate with pK_a reflect and can be used to study the specificity of the enzyme (Hinkle & Kirsch, 1971; Schechter & Berger, 1968; Fink & Bender, 1969). Although this procedure for examining the specificity of the S₁' subsite of papain obviates the need to synthesize suitable synthetic

peptides, the results must be treated with caution since clearly such nucleophiles could bind at other subsites in the active site and so influence the observed kinetic parameters. Fink & Bender (1969) observed even with *n*-alkyl alcohols that only methanol and ethanol gave kinetic data consistent with the partitioning of the acyl-enzyme between the alcohol and water. With higher alcohols, the kinetic evidence was consistent with a scheme in which the alcohol binds to the enzyme in at least two different modes.

Inspection of the model proposed for the enzyme-substrate complex with residues bound in the S_1 and S_2 subsites (Lowe & Yuthavong, 1971a; Wolthers *et al.*, 1970) suggested that the S_1' subsite might consist of the hydrophobic region near tryptophan-177 and alanine-136. These residues would be expected to provide the necessary micro-environment for binding hydrophobic residues. The possibility that this subsite might extend as far as glutamine-19 suggested that the subsite might provide hydrogen-bonding capability for certain side chains. An investigation of a number of L- α -amino acid amides for the nucleophile-assisted deacylation of *N*-benzyloxycarbonylglycyl-papain was undertaken, but for reasons already indicated and because it was desirable to know whether the specificity of the S_1' subsite was expressed in binding or catalysis, a number of peptides with the general structure *N*-benzyloxycarbonylglycyl L-amino acid amide were synthesized and their kinetic parameters determined. The *N*-benzyloxycarbonyl group, which is structurally similar to phenylalanine (Lowe, 1970), was expected to bind in the S_2 subsite and so place the C-terminal amino acid amide in the S_1' subsite.

Materials and Methods

Materials

Deionized and deaerated water was used throughout. Methanol was dried with magnesium as described by Vogel (1964) and distilled. Thionyl chloride was distilled and the fraction of b.p. 75.5–76.0°C used.

Benzyl chloroformate, glycine ethyl ester hydrochloride, sodium sulphite, sodium acetate trihydrate (A.R.) and mercaptoethanol were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. L-Aspartic acid, glycine amide hydrochloride, L-cysteine hydrochloride and L-leucine amide hydrochloride were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. L-Valine amide hydrochloride and L-leucine amide were obtained from Micro-Bio Laboratories Ltd., London W.11, U.K. L-Tryptophan amide hydrochloride and *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. *N*-Benzyloxycarbonylglycyl-L-valine amide was obtained from Cyclo Chemicals, via Cambrian Chemicals Ltd.,

Croydon, Surrey, U.K. Dimethylformamide, ethyl acetate (A.R.), EDTA dihydrate (A.R.) and HgCl_2 were obtained from Fisons Ltd., Loughborough, Leics., U.K. Light petroleum (b.p. 40–60°C) was distilled. *N*-Benzyloxycarbonylglycine picryl ester and L-alanine picryl ester were gifts from Dr. G. T. Young, University of Oxford, U.K.

Papain. Twice-crystallized papain was obtained from dried papaya latex (we acknowledge a generous gift of this material from the Wallerstein Co., Morton Grove, Ill., U.S.A.) by the method of Kimmel & Smith (1954) and was purified to 100% papain either by the method of Sluyterman & Widjenes (1970) or by the method of Blumberg *et al.* (1970).

Activation of mercuri-papain. Mercuri-papain was activated by treatment for 1 h at room temperature with 2-mercaptoethanol present in approx. 1000-fold molar excess in the presence of EDTA (approx. 1 mM) at pH 5. The resulting solution was diluted to the required concentration with 1 mM-EDTA before assaying the enzymic activity. Solutions prepared in this way, with a final extinction of less than 5 at 278 nm, showed no loss of activity over several hours if kept at 0°C.

Analytical methods

Melting points were determined with a Kofler hot-stage apparatus. Optical rotations were measured with a Perkin-Elmer 141 photoelectric polarimeter at 20°C for solutions in a 0.1 m cell. Microanalyses were determined by Dr. F. B. Strauss and his staff in this laboratory.

Other methods

Assay of papain activity. The spectrophotometric assay proposed by Kirsch & Ingelström (1966) was used with *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester as substrate.

The reaction was carried out in a phosphate buffer at pH 6.0, consisting of 100 mM-sodium phosphate and 1 mM-EDTA. Enzyme solution (10 μl) was added to 3.0 ml of this buffer (maintained thermostatically at 25°C in a 1 cm cuvette in a Unicam SP. 1800 spectrophotometer) to give an enzyme concentration of about 100 nM.

The reaction was initiated by addition of 5 mM-*N*-benzyloxycarbonylglycine *p*-nitrophenyl ester solution in acetonitrile (50 μl), and the absorption at 340 nm (due to *p*-nitrophenol) plotted automatically as a function of time.

K_m for this substrate under these conditions was 8.3 μM (Kirsch & Ingelström, 1966), and the substrate concentration in this assay was approx. 78 μM . All measurements were related to $k_{\text{cat}} = 8.0 \text{ s}^{-1}$ at 25°C for the papain-catalysed hydrolysis of *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester.

Nucleophile-assisted deacylations of *N*-benzyloxycarbonylglycyl-papain. These reactions were per-

formed at pH 7.5 and 35°C in a sodium phosphate buffer (32mM) containing 1mM-EDTA. The amine hydrochloride was dissolved in the phosphate buffer (approx. 7ml) at 35°C, the pH adjusted to 7.5 and the solution made up to 20ml with sodium phosphate buffer. Reaction mixtures were made up in 1cm path-length cuvettes by mixing known volumes of nucleophile solution and phosphate buffer, to a constant volume of 2.5ml. The cuvettes were warmed to 35°C in the thermostatically controlled spectrophotometer. The enzyme (10 or 25 μ l of E_{278} approx. 0.7) was added and the reaction initiated by addition of substrate, *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester, in acetonitrile solution. To approach $V_{\max}$, conditions the substrate concentration used was near the solubility limit under these conditions.

The procedure used to follow spontaneous hydrolysis was similar except that instead of enzyme solution, a solution containing 2-mercaptoethanol and EDTA only (at the same concentrations) was added. Any differences between the results could therefore be attributed to the enzyme. No pH change was detected after enzymic hydrolysis. The progress curves of the reactions were recorded directly on paper tape by using a Stogate data logger and $V_{\max}$ and K_m parameters were calculated by using a program which allows direct non-linear least-squares fitting to the integrated rate equation for decay of substrate concentration.

Under these conditions the minimal three-step reaction pathway must be expanded to:

Since P_1 is being followed:

$$V_{\max} = (k_3' + k_4N)k_2E_0/(k_2 + k_3' + k_4N)$$

where N is the concentration of added nucleophile and E_0 the concentration of enzyme.

Expanding $V_{\max}$ as a binomial series in N gives:

$$V_{\max} = k_2E_0 \left(\frac{k_3' + k_4N}{k_2 + k_3'} \right) \left(1 - \frac{k_4N}{k_2 + k_3'} + \dots \right)$$

By using a nucleophile concentration such that $k_4N \ll k_2$:

$$V_{\max} = \frac{k_2E_0}{k_2 + k_3'} \left(k_3' + \frac{k_2k_4N}{k_2 + k_3'} \right)$$

A plot of $V_{\max}$ against N gives a straight line and the ratio of the slope to the intercept is proportional to k_4 . The relative rate constants for different L-amino acid amides can therefore readily be determined.

Hydrolysis of amide substrates. A Radiometer pH-stat (type TTT1c) and recorder (type SBR 2c) equipped with a burette assembly and thermostat

was used. A solution containing 0.3M-NaCl and 1mM-EDTA in water, or in aq. 20% (v/v) dimethylformamide, was used as solvent. The substrate was dissolved and the volume made up to 20.0ml with the same solvent. Reaction mixtures of various substrate concentrations were made up by mixing these two solutions, to a constant volume of 5.0ml, in 10ml reaction vessels. A combination electrode (type GK2321c) was lowered into the stirred solution, and the whole assembly left for several minutes to reach thermal equilibrium at 35°C. The pH was adjusted near 7.5 by addition of small quantities of 1M-NaOH or 10M-HCl. During the reaction a gentle stream of N_2 was passed over the solution.

Reaction was initiated by addition of papain solution (100 or 200 μ l of approx. E_{278} 30). In no case was there spontaneous (non-enzymic) hydrolysis before addition of enzyme.

For each run the initial rate was measured by drawing a tangent to the experimental curve. Values of $V_{\max}/K_m$ were calculated from the slope of a Lineweaver-Burk plot by using a linear least-squares fitting of the experimental data. The best-fit values of $V_{\max}$ and K_m and their standard errors were calculated on a Hewlett-Packard programmable calculator by the method of Wilkinson (1961).

Papain-catalysed hydrolysis of *N*-benzyloxycarbonylglycine amide. A 50mM solution of this substrate was made up in the aq. 20% (v/v) dimethylformamide solvent at pH 7.5 with an ionic strength of 0.3M. A 5ml portion of this solution was placed in a reaction vessel maintained at 35°C and stirred for 10min to establish thermal equilibrium. A solution of 0.5M-2-mercaptoethanol in aq. 20% (v/v) dimethylformamide (0.05ml), containing 0.3M-NaCl and 1mM-EDTA, was added to the reaction mixture. An enzyme solution was prepared in aq. 20% (v/v) dimethylformamide containing NaCl (0.3M) and EDTA (1mM) and warmed to 35°C before use. A portion (1ml) of this solution was added to the reaction mixture. When the enzyme was added, and at 4min intervals thereafter, a portion (0.2ml) was withdrawn from the reaction vessel. These samples were treated with 2.5ml of 1% ninhydrin solution containing a little stannous chloride and sodium acetate buffer (2ml, pH 5.3). The stannous chloride inactivates the enzyme preventing further hydrolysis of the substrate (Smith *et al.*, 1958). The samples were incubated at 100°C in a boiling-water bath for 10min in stoppered tubes and then allowed to cool to room temperature. The extinction at 570nm was measured for each sample. The sample taken when the enzyme was added served as a blank solution. Samples were taken from the reaction mixture up to 40min after the addition of the enzyme and no significant departure was observed from the blank value of the extinction. The hydrolysis of 1% of the substrate would be readily detected by the procedure used.

Experiments to establish the site of papain-catalysed cleavage of the substrates. About 10 μ mol of the substrate was incubated with papain at 40°C until 90% hydrolysis had taken place. An enzyme concentration of about one-thousandth of that of the substrate was used. The solution was deproteinated by passage through an Amicon PM-10 ultrafiltration membrane and hydrogenated overnight in the presence of 5% palladium on charcoal. A sample of the solution was examined on a JEOL JLC-5AH amino acid analyser.

Measurement of pK_a values. pK_a values were required under the specified experimental conditions [i.e. 35°C, 0.3M-NaCl, water or aq. 20% (v/v) dimethylformamide]. A Radiometer pH-meter and recorder were used in the titration mode. The electrode was calibrated in the region of the pK_a found.

Preparation of N-benzyloxycarbonyl-glycylglycine ethyl ester. N-Benzyloxycarbonylglycine (1.34 g, 6.4 mmol) (Greenstein & Winitz, 1961a) and dicyclohexylcarbodi-imide (1.40 g, 6.8 mmol) were dissolved in dimethylformamide (20 ml) and cooled to 0°C. The solution was stirred for 15 min at 0°C, triethylamine (0.95 ml, 6.6 mmol) and glycine ethyl ester hydrochloride (0.87 g, 6.7 mmol) were added and the resulting mixture was stirred at room temperature overnight. The precipitate was filtered off and the solvent removed on a rotary evaporator. Ethyl acetate was added to dissolve the peptide and the resulting mixture filtered to remove dicyclohexylurea. The filtrate was washed successively with 2M-HCl, a saturated NaHCO₃ solution and water. The organic layer was dried over MgSO₄, the solvent removed in a rotary evaporator and the residual solid redissolved in the minimum quantity of ethyl acetate. The product was carefully precipitated by the addition of light petroleum (b.p. 40–60°C). The solid was again precipitated from solution in ethyl acetate by the addition of light petroleum to give N-benzyloxycarbonyl-glycylglycine ethyl ester (0.93 g), m.p. 81°C (literature m.p. 82°C, Goldschmidt & Wick, 1952; Goldschmidt & Lautenschlager, 1953).

Preparation of N-benzyloxycarbonyl-glycyl-L-leucine methyl ester. N-Benzyloxycarbonylglycine (1.34 g, 6.4 mmol) and dicyclohexylcarbodi-imide (1.37 g, 6.7 mmol) were dissolved in 20 ml of dimethylformamide and the solution was stirred for 15 min at 0°C. Then L-leucine methyl ester hydrochloride (1.21 g, 6.7 mmol) (Brenner & Huber, 1953) and triethylamine (0.90 ml, 6.2 mmol) were added and the resulting mixture was stirred at room temperature overnight. The concentration of triethylamine used was less than that of the L-leucine methyl ester hydrochloride to ensure that no base-catalysed racemization of the dipeptide took place. After filtering off the dicyclohexylurea, the solvent was removed in a rotary evaporator. The residue was dissolved in ethyl acetate

and washed successively with 2M-HCl, a saturated NaHCO₃ solution and water before being dried over MgSO₄. The ethyl acetate was removed, giving an oil which could not be induced to crystallize.

Preparation of N-benzyloxycarbonyl-glycyl-L-tryptophan methyl ester. N-Benzyloxycarbonyl-glycyl-L-tryptophan methyl ester was prepared and purified in the same way as the leucine derivative, again ensuring that there was a slight deficiency of triethylamine, so that base-catalysed racemization did not take place. The product was obtained as an oil; Würz *et al.* (1962) have reported that they were unable to crystallize this compound.

Preparation of N-benzyloxycarbonyl-glycyl-L-aspartic acid dimethyl ester. To a stirred suspension of N-benzyloxycarbonylglycine (4.18 g, 20 mmol) and L-aspartic acid dimethyl ester (3.93 g, 20 mmol) (Brenner & Huber, 1953) in sodium-dried tetrahydrofuran (75 ml), was added 1-hydroxybenzotriazole (5.4 g, 40 mmol) (König & Geiger, 1970). The mixture was cooled to 0°C and triethylamine (2.78 ml, 20 mmol) was added. Finally, dicyclohexylcarbodi-imide (4.33 g, 21 mmol) dissolved in tetrahydrofuran (30 ml) was added at 0°C and the mixture was stirred for 1 h at 0°C and overnight at room temperature. The precipitate of dicyclohexylurea was filtered off, and the solvent was removed from the filtrate leaving a thick oil, which was taken up in ethyl acetate, dried over MgSO₄ and kept at 0°C for several hours to complete the precipitation of the urea. After filtering and removing the solvent, the residual oil (6.77 g) was taken up in dimethylformamide and water added dropwise until an oil (approx. 2 g) separated which was shown to be pure by t.l.c.

Preparation of N-benzyloxycarbonyl-glycyl-L-glutamic acid dimethyl ester. N-Benzyloxycarbonylglycine and L-glutamic acid dimethyl ester hydrochloride (Rachele, 1963; Zahn *et al.*, 1963) were coupled by using 1 equiv. of dicyclohexylcarbodi-imide and 2 equiv. of 1-hydroxybenzotriazole as in the preparation of N-benzyloxycarbonyl-glycyl-L-aspartic acid dimethyl ester. The product was obtained as a pure oil in 95% yield.

Amination. The peptide esters were all converted into the corresponding amides by the following procedure, adapted from the method of Würz *et al.* (1962). Anhydrous methanol (25 ml) was cooled to 0°C and saturated with NH₃. The saturated solution was then added to the peptide ester and the mixture kept in a well-stoppered flask for 2 days. The purification of each product is described below.

N-Benzyloxycarbonyl-glycylglycine amide. The solvent was removed from the methanolic solution in a rotary evaporator and the residual solid recrystallized from methanol to give N-benzyloxycarbonyl-glycylglycine amide (0.63 g), m.p. 177–178°C (Jeschkeit, 1968; m.p. 179–180°C) (Found: C, 54.4; H, 5.7;

N, 15.8. Calc. for C₁₂H₁₅N₃O₄: C, 54.4; H, 5.7; N, 15.8%.

N-Benzoyloxycarbonylglycyl-L-leucine amide. The solvent was removed from the methanolic solution in a rotary evaporator. The residue was triturated with light petroleum (b.p. 40–60°C) to give the peptide (1.10 g), m.p. 122–123°C, $[\alpha]_D^{20}$ –18.8° (Smith & Slonim, 1948; m.p. 123–124°C) (Found: C, 59.6; H, 7.3; N, 12.9. Calc. for C₁₆H₂₃N₃O₄: C, 59.8; H, 7.2; N, 13.1%).

N-Benzoyloxycarbonylglycyl-L-tryptophan amide. After removal of the solvent, the residue was dissolved in ethyl acetate and precipitated by the addition of light petroleum. The solid was filtered off and washed with small quantities of ice-cold methanol. The product had m.p. 160–161°C, with softening at 117–123°C. Softening at this temperature was observed by Smith & Slonim (1948) with a final melting point at 145°C. Since this compound has also been synthesized with melting points of 163°C and 169–170°C respectively (Würz *et al.*, 1962; Theodoropoulos & Fruton, 1962), it seems likely that an impurity caused the softening at 117–123°C and also the low melting point. Recrystallization from methanol–diethylether (~1:1, v/v) gave the peptide (0.75 g), m.p. 168–169°C, $[\alpha]_D^{20}$ +2.0° (Found: C, 63.8; H, 5.6; N, 14.0. Calc. for C₂₁H₂₂N₄O₄: C, 63.9; H, 5.6; N, 14.2%).

N-Benzoyloxycarbonylglycyl-L-asparagine amide. After aminolysis of the dimethyl ester for 3 days, *N*-benzyloxycarbonylglycyl-L-asparagine amide had crystallized in the reaction vessel. The solid was collected and a second crop of crystals was obtained by removing all the solvent at room temperature and recrystallizing from methanol–ether (~1:2, v/v). The pure product (2.06 g) had m.p. 185–187°C, $[\alpha]_D^{20}$ –1.0 (c 0.6 in dimethyl sulphoxide) (Found: C, 52.1; H, 5.6; N, 17.3; C₁₄H₁₈N₄O₅ requires C, 52.2; H, 5.6; N, 17.4%).

N-Benzoyloxycarbonylglycyl-L-glutamine amide. After aminolysis of the dimethyl ester for 3 days the solvent was removed and the product crystallized from methanol–water (~1:1, v/v) (over 7 days) to give *N*-benzyloxycarbonylglycyl-L-glutamine amide (2.7 g), m.p. 195–197°C, $[\alpha]_D^{20}$ –0.7° (c 0.85 in dimethyl sulphoxide) (Found: C, 53.4; H, 5.9; N, 16.5; C₁₅H₂₀N₄O₅ requires C, 53.6; H, 6.0; N, 16.7%).

N-Benzoyloxycarbonylglycine amide. This compound was prepared by the method of Camble *et al.* (1969)

and had m.p. 136–137°C (literature m.p. 138°C) (Found: C, 57.4; H, 5.7; N, 13.4; C₁₀H₁₂N₂O₃ requires C, 57.7; H, 5.8; N, 13.5%).

L-Asparagine amide hydrochloride. After aminolysis of L-aspartic acid dimethyl ester hydrochloride (Brenner & Huber, 1953), the solvent was removed and the product crystallized from methanol–water–ether (~3:3:1, by vol.) as needles (1.41 g), m.p. 215–217°C (decomp.), $[\alpha]_D^{20}$ +12° (c 1 in water) (Smith & Spackman, 1955; m.p. 214–215°C; $[\alpha]_D^{20}$ +15.8; c 1 in water).

L-Glutamine amide hydrochloride. *N*-Benzoyloxycarbonylglutamic acid dimethyl ester (6.8 g) was prepared as described by Greenstein & Winitz (1961b). During the aminolysis (for 5 days) the product precipitated spontaneously. The product was filtered and washed with methanol. The filtrate was evaporated to give a further crop of product. Recrystallization of all the material from ethanol gave *N*-benzyloxycarbonylglutamine amide (5.4 g) as needles, m.p. 198–201°C, $[\alpha]_D^{20}$ 5.27° (c 2 in dimethylformamide) (Fruton, 1946; Meyers *et al.*, 1969; m.p. 195–197°C; $[\alpha]_D^{20}$ 10.3°).

N-Benzoyloxycarbonylglutamine amide (1.97 g) was dissolved in methanol–water (~2:1, v/v) (approx. 60 ml), 5% palladium on charcoal (0.2 g) and 1 equiv. of HCl was added, and the mixture was shaken under H₂ at room temperature and atmospheric pressure for 6 h. Uptake of H₂ ceased after 90 min. The catalyst was filtered off and the solvent removed. The product was purified by two precipitations from water by addition of ethanol to give needles (1.03 g), m.p. 184–186°C $[\alpha]_D^{20}$ = 19.7° (c 1 in water) (Meyers *et al.*, 1969; m.p. 193–196°C; $[\alpha]_D^{25}$ 20.1°) (Found: C, 32.8; H, 6.8; Cl, 20.0; N, 23.4. Calc. for C₅H₁₂ClN₃O₂: C, 33.1; H, 6.7; Cl, 19.5; N, 23.1%).

Results and Discussion

Evidence in the literature suggests that the S₁' subsite in papain has the ability to bind hydrophobic residues (Brubacher & Bender, 1966; Lowe & Yuthavong, 1971b; Williams *et al.*, 1972a,b), but no systematic study of the specificity of this subsite has previously been undertaken. Accordingly, a range of amino acid amides was selected to investigate their ability to enhance the rate of deacylation of acyl-papain derived from *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester. The dissociation constants of

Table 1. Dissociation constants of some amino acid amides

The dissociation constants were determined potentiometrically at 35°C in 0.3 M-NaCl.

	GlyNH ₂	AsnNH ₂	GlnNH ₂	ValNH ₂	TrpNH ₂	LeuNH ₂
pK _a in water	8.08	6.98	7.38	7.71	7.52	7.81
pK _a in aq. 20% (v/v) dimethylformamide	7.93	6.96	7.43	7.79	7.52	7.68

Table 2. Relative rate constants for the deacylation of *N*-benzyloxycarbonylglycyl-papain by amino acid amides (normalized to L-valine amide) at pH 7.5 and 35°C in 0.3M-NaCl solution

For details see the text.						
Relative <i>k</i>	GlyNH ₂	AsnNH ₂	GlnNH ₂	ValNH ₂	TrpNH ₂	LeuNH ₂
	0.13 ± 0.04	0.07 ± 0.01	0.50 ± 0.05	1 ± 0.1	2 ± 0.1	11 ± 4

Table 3. Michaelis-Menten parameters for the papain-catalysed hydrolysis of some *N*-benzyloxycarbonylglycyl-amino acid amides at 35°C and pH 7.5 in 0.3M-NaCl in aq. 20% (v/v) dimethylformamide

The enzyme concentration was 1–50 μM, depending on the substrate. The data for k_{cat} are relative to a value of 8.0 s⁻¹ for *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester at 25°C. Abbreviation: Z, Benzyloxycarbonyl.

Substrate	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ ·s ⁻¹)	No. of points	Substrate concn. (mM)
Z-Gly-GlyNH ₂	0.08 ± 0.02	13.0 ± 5.5	9	2.0–7.8
Z-Gly-AsnNH ₂	0.08 ± 0.01	8.6 ± 0.3	11	3.0–15.1
Z-Gly-GlnNH ₂	0.06 ± 0.01	10.5 ± 1.7	5	0.7–2.4
Z-Gly-ValNH ₂	0.39 ± 0.05	107 ± 4	6	0.5–2.5
Z-Gly-LeuNH ₂	1.29 ± 0.19	660 ± 68	7	0.9–9.4
Z-Gly-TrpNH ₂	0.73 ± 0.12	815 ± 110	8	2.2–6.5

these amino acid amides were measured under the conditions used in the kinetic experiments (Table 1).

The effect of the amino acid amides on the kinetic parameters of the papain-catalysed hydrolysis of *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester was determined at pH 7.5 and 35°C and the concentration of unprotonated nucleophile calculated from the data in Table 1. The production of *p*-nitrophenol was followed spectrophotometrically at 370 nm. The relative rate constants for the deacylation of *N*-benzyloxycarbonylglycyl-papain by each L-amino acid amide are shown in Table 2. Although these results must be treated with caution for the reasons already expressed and because the intrinsic nucleophilicities of the amino acid amides will be related to their dissociation constants which vary over more than 1 pH unit, it is nevertheless apparent that the S₁' subsite has a preference for hydrophobic residues and there is no evidence from these data for hydrogen-bonding ability in this subsite, at least to L-asparagine amide or L-glutamine amide. The rather low value for L-asparagine amide is, of course, likely to be due, in part at least, to its relatively poor nucleophilicity, its p*K*_a being 0.4 unit below that of L-glutamine amide and 1.1 units below that of glycine amide in water.

The *N*-benzyloxycarbonylglycyl amino acid amides shown in Table 3 were prepared by conventional methods of peptide synthesis, and the papain-catalysed hydrolysis was followed by pH-stat at pH 7.5 and in 20% dimethylformamide. The organic solvent was necessary to obtain the required concentration of *N*-benzyloxycarbonylglycyl-L-tryptophan amide and, for comparison, kinetic parameters for the other substrates were determined in this sol-

vent. The dissociation constants of the amino acid amides in this solvent (Table 1) were used to calculate the extent of cleavage from the amount of alkali required to maintain constant pH. The products of the papain-catalysed hydrolysis were investigated after more than 90% cleavage had occurred. Deproteination of the reaction mixture was followed by catalytic hydrogenation of the *N*-benzyloxycarbonyl group and the reaction mixture was analysed. For *N*-benzyloxycarbonylglycylglycine amide, glycine, glycine amide and a trace of glycylglycine amide were detected, but no glycylglycine. For *N*-benzyloxycarbonylglycyl-L-leucine amide, glycine, leucine amide and a trace of glycyl-leucine amide were detected, but no glycyl-leucine. Hence with these substrates for which the enzyme has rather different overall rates of cleavage, the point of cleavage, as expected, is dominated by the benzyloxycarbonyl group.

The most reliable measure of enzyme specificity is k_{cat}/K_m since, if non-productive binding occurs, it does not influence this expression (Bender & Kézdy, 1965). The data indicate (Table 3) that the side chains of L-asparagine amide and L-glutamine amide do not facilitate cleavage, their overall rates (k_{cat}/K_m) being similar to that for *N*-benzyloxycarbonylglycylglycine amide. The fact that *N*-benzyloxycarbonylglycine amide is not hydrolysed at all under comparable conditions implies that the terminal amide makes a significant contribution to binding and/or catalysis. This is particularly interesting in view of the very important role played by the peptide group on the *N*-terminal side of the peptide bond to be cleaved (Lowe & Yuthavong, 1971a), and indicates that the main specificity of this enzyme is for the peptide

backbone of the substrate. This specificity can of course be markedly enhanced by the presence of appropriate side chains.

Since k_{cat} for *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester (8.0s^{-1}) is considerably greater than any of the k_{cat} values observed for the peptide substrates in Table 3, k_{cat} for these substrates represents the acylation constant, k_2 . The data in Table 3 show that the specificity for the hydrophobic residues L-valine, L-leucine and L-tryptophan is distributed between the binding and acylation steps. Thus the specificity of the S₁' subsite of papain manifests itself in part in the acylation step, as has been found for other specificity sites of the enzyme so far investigated (Lowe & Yuthavong, 1971a).

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