Biochem. J. (1974) **141**, 503–515
Printed in Great Britain

A Kinetic and Fluorimetric Investigation of Papain Modified at Tryptophan-69 and -177 by *N*-Bromosuccinimide

By GORDON LOWE and ALAN S. WHITWORTH
*The Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford OX*1 3*QY, U.K.*

(*Received* 10 *January* 1974)

A systematic study of the modification of papain (its thiol group protected as a disulphide with mercaptoethanol) by *N*-bromosuccinimide, showed that 2 molar equiv. modified tryptophan-69 and 4 molar equiv. modified tryptophan-69 and -177. The Michaelis parameters for the catalysed hydrolysis of *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester by these modified enzymes were determined. The enzymic activity of the modified enzymes was not seriously impaired, but modification of tryptophan-177 raised the apparent pK_a of the acidic limb of the pH profile by more than 1 pH unit for both $k_{cat.}$ and $k_{cat.}/K_m$. The fluorescence spectra (excitation at 288nm) of the modified enzymes showed that tryptophan-69 contributed about 8% to the fluorescence intensity, whereas tryptophan-177 contributed about 46% at neutral pH. However, the contribution of tryptophan-177 was quenched at low pH and its fluorescence intensity showed sigmoidal pH-dependence, with an apparent pK_a of 4.2. Histidine-159, which is in close contact with tryptophan-177, is considered to be the residue responsible for the fluorescence quenching. When tryptophan-177 was modified, presumably generating a less hydrophobic micro-environment, the apparent pK_a determined kinetically was raised to about 5.4. By comparing the Michaelis parameters of native papain, papain modified at tryptophan-69 and papain modified at tryptophan-69 and -177 with *N*-benzyloxycarbonylglycylglycine amide and *N*-benzyloxycarbonylglycyltryptophan amide, tryptophan-69 and tryptophan-177 were shown to be structural features of the S_2 and S_1' subsites respectively.

The proteolytic enzyme, papain, is capable of accommodating in its active site up to seven amino acid residues of a peptide substrate, four residues on the *N*-terminal side of the point of cleavage (subsites S_1–S_4) and three residues on the *C*-terminal side (subsites S_1'–S_3') (Berger & Schechter, 1970). Kinetic studies on the specificity of the enzyme for the peptide linkage between the amino acid residues bound in subsites S_1–S_2 and the side chain of the amino acid residue in subsite S_2 have allowed a model to be built of the enzyme–substrate complex for the residues bound in the S_1 and S_2 subsites (Lowe & Yuthavong, 1971). From the low-resolution [0.6nm (6Å)] X-ray-crystallographic data for papain inhibited with *N*-t-butoxycarbonyl-*p*-iodophenyl-alanyl-leucine (Wolthers *et al.*, 1970), in which the position of the I atom could be determined, a model of the papain–peptide complex was built which was in general agreement with that based on specificity studies. The model shows that the S_2 subsite is lined by a number of hydrophobic residues, amongst which is tryptophan-69. Of the five tryptophan residues (7, 26, 69, 177, 181) in papain (Husain & Lowe, 1969; Mitchel *et al.*, 1970) tryptophan-69 and -177 appear in the crystal structure to be the most exposed (J. Drenth, personal communication) and in principle therefore should be capable of selective chemical modification.

The role of tryptophan-177 is particularly interesting because it appears from the crystal structure to partially shield the active-site imidazole group of histidine-159 from the solvent water and hence to be responsible in part for the abnormally low pK_a of this residue (Allen & Lowe, 1973; Williams *et al.*, 1972; Polgar, 1973). Further, the model for the binding of peptides in the S_1 and S_2 subsites (Lowe & Yuthavong, 1971) suggested that if a hydrophobic amino acid was bound in the S_1' subsite it would probably interact with the indole ring of tryptophan-177. This subsite has an affinity for hydrophobic amino acid residues, especially tryptophan and leucine (Alecio *et al.*, 1974).

The selective oxidation of the indole rings of tryptophan-69 and -177, followed by specificity studies of the modified enzyme, should provide evidence for the presence of these residues in the S_2 and S_1' subsites and also for the hypothesis that the abnormally low value of the pK_a for active-site

histidine-159 is due to its hydrophobic environment.

One of the most widely used reagents for the modification of tryptophan residues is *N*-bromosuccinimide which with an intact enzyme generally oxidizes the indole to an oxindole ring and may subsequently brominate it. It is known, however, that this reagent is capable of modifying cysteine, methionine, tyrosine and histidine residues (Perham, 1969). There have been several reports in recent years of the use of *N*-bromosuccinimide for the modification of papain (Sun & Tsou, 1963, 1965; Steiner, 1971; Kirschenbaum, 1971; Löffler & Schneider, 1972). This literature contains conflicting statements, however, some studies indicating that the activity of the enzyme is enhanced after modification with *N*-bromosuccinimide (Kirschenbaum, 1971; Löffler & Schneider 1972), whereas others claim that it is lost (Sun & Tsou, 1963, 1965). Since none of these studies have been coupled with a structural investigation to establish exactly what chemical modifications have been achieved or how selective they were, it seems likely that the modified proteins obtained were mixtures of several species. It is difficult therefore to assess the meaning of these statements.

By contrast proflavin-sensitized photo-oxidation of papain selectively modifies only tryptophan residues and under carefully defined conditions below 15°C tryptophan-69 alone can be photo-oxidized to formylkynurenine, whereas above this temperature both tryptophan-7 and tryptophan-69 are converted into formylkynurenine (Jori & Galiazzo, 1971). Modification of these residues caused a 25% decrease in enzymic activity towards *N*-α-benzoyl-L-arginine ethyl ester; unfortunately no more detailed kinetic analysis was undertaken.

By lowering the pH or raising the temperature the proflavin-sensitized photo-oxidation of tryptophan-7, -69 and -177 could be achieved (Jori & Galiazzo, 1971). Since the additional conversion of tryptophan-177 into formylkynurenine led to essentially complete loss of enzymic activity towards *N*-α-benzoyl-L-arginine ethyl ester with apparently only minor changes in the papain conformation, it was concluded that the formylkynurenine in position 177 inhibits the enzymic activity towards this substrate, and the suggestion was made that because of the proximity of tryptophan-177 to the active-site cysteine-25 and histidine-159, this residue might conceivably participate directly in the enzymic activity of the enzyme.

In spite of the many reports in which tryptophan residues of papain have undoubtedly been modified, no evidence of the effect of the modification of tryptophan-177 on the pK_a of the imidazole group of histidine-159 has been published. Likewise the effect of oxidation of tryptophan-69 and -177 on more specific substrates has not been investigated.

Experimental conditions have now been found for the selective oxidation of tryptophan-69 and of tryptophan-69 and tryptophan-177. The fluorescence and enzymic properties of these modified enzymes after purification have been investigated.

Materials and Methods

Materials

Unless otherwise stated, all materials were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. and were of the highest grade available. Acetonitrile was purified by treatment with anhydrous Na_2CO_3 and then redistilled from phosphoric oxide (b.p. 80.5–81.0°C). Iodoacetic acid was recrystallized from light petroleum (b.p. 100–120°C) and stored in the dark. Guanidinium chloride (BDH Chemicals Ltd., biochemical grade) was recrystallized twice from methanol. *N*-Bromosuccinimide was recrystallized from water and the crystals were dried over phosphoric oxide *in vacuo* in the dark (Found: C, 27.1; H, 2.2; N, 7.8; Br, 44.5; Calc. for $C_4H_4NO_2Br$, C, 27.0; H, 2.38, N, 7.98; Br, 44.98%).

2-Hydroxyethyldisulphide. I_2 (7g) dissolved in ethanol (50ml) was slowly added to a stirred solution of 2-mercaptoethanol (4ml) in aqueous ethanol (50ml; 50%, v/v) until a permanent pink colour was obtained. The solution was decolourized by the addition of one drop of 2-mercaptoethanol, adjusted to pH7 with 4M-NaOH solution and evaporated to dryness. The residue was extracted with boiling chloroform (3×100ml), the solvent removed from the extract under reduced pressure and the residue distilled to give 2-hydroxyethyl disulphide (1.9g, 43.5%), b.p. 110–112°C at 10.6 Pa (0.08mmHg).

Analytical methods

Amino acid analyses were determined with a JEOL JLC-5AH amino acid analyser. U.v. spectra and absorbance values at particular wavelengths were recorded on a Unicam SP. 1800 spectrophotometer.

pH measurements and pH-stat work. A Radiometer pH meter (type TTTlc) was used with a combination electrode (type GK 2321c) which had been standardized against a BDH pH4.00 phthalate buffer (BDH Chemicals Ltd.), at the temperature of the experiment. For use as a pH-stat the meter was coupled to a Radiometer titrigraph recorder (type SBR2c) and a syringe burette which delivered 0.5ml at full-scale deflexion of the recorder pen.

Measurement of protein concentration. The concentration of unmodified papain solutions, whether active or inactivated as mercuri-papain or papain-SS-C_2H_4OH, was calculated by using $\varepsilon = 58500$ litre·mol^{-1}·cm^{-1} at 280nm, on the basis of a molecular weight of 23406 (Mitchel *et al.*, 1970) and $E_{872}^{1\%} = 25$

(Glazer & Smith, 1961). For the *N*-bromosuccinimide-modified papain species, the change in u.v. spectrum upon modification was recorded, which enabled the extinction coefficient of the new species to be calculated.

Concentration, ultrafiltration and dialysis of protein solutions. Ultrafiltration cells (type 202 and 12, Amicon Ltd., High Wycombe, Bucks., U.K.) with Diaflo membranes (type PM-10) were used to concentrate papain solutions and to change the buffer medium of such solutions. When protein precipitation was expected, normal dialysis with Visking tubing was used. Before use the tubing was boiled for 30min in 10mM-EDTA solution and washed with water.

Fluorescence spectra. The fluorescence emission spectra of the papain-SS-C_2H_4OH before and after treatment with *N*-bromosuccinimide were recorded at room temperature with a Perkin–Elmer–Hitachi spectrophotofluorimeter (model MPF-2A) with an excitation wavelength of 288nm. The sample (2.5ml) contained protein (approx. 1.2μM) in McIlvaine's phosphate–citrate buffer (0.3M-Na^+, 1mM-EDTA) in the pH range 2.5–8.0 (Dawson *et al.*, 1969*a*), or in glycine–NaOH buffer (0.1M-glycine, 0.2M-NaCl, 1mM-EDTA) in the pH range 8.0–11.0, and the pH of each sample was checked immediately after recording its fluorescence spectrum. The components of the buffer made a negligible contribution to the total fluorescence of the protein solution. The intensity of emission of each sample was read at its $\lambda_{max.}$ and after correction for slight concentration differences was expressed relative to that of a sample of unmodified papain-SS-C_2H_4OH at pH6.90, recorded at the same time.

Amino acid analysis. Papain or peptide solution (50nmol) was freeze-dried in a hydrolysis tube and 6M-HCl (1ml) containing 0.5% phenol was added. The tube was chilled in an acetone–solid CO_2 bath, evacuated to de-gas the solution, flushed twice with N_2 and sealed under vacuum. After hydrolysis at 115°C for 24h the solution was evaporated at room temperature over KOH pellets in an evacuated desiccator and the residue dissolved in 0.01M-HCl (2.5–5.0ml) for analysis.

Tryptophan analysis. Since neither the method of Matsubara & Sasaki (1969) nor that of Liu & Chang (1971) gave 100% recovery of tryptophan and the loss on hydrolysis was not consistent enough to use a routine correction, a colorimetric method of tryptophan determination was used, adapted from that of Graham *et al.* (1947).

Papain (0.2ml; 0.1–0.15mM) in water or dilute buffer (less than 20mM) was incubated in a stoppered tube with 5M-NaOH (50μl) containing gelatin (7%, w/v) at 100°C for 2h and left to cool. Then 12M-HCl (2.5ml), 4-dimethylaminobenzaldehyde (50μl; 2.5%, w/v, in 2M-H_2SO_4) and sodium nitrate (25μl; 2%, w/v, in 2M-H_2SO_4) were added, mixed and kept for 30min. Aqueous ethanol (7.0ml; 50%, v/v) was added, mixed and the absorbance at 610nm recorded.

A calibration graph was constructed with *N*-acetyl-DL-tryptophan solutions of known concentration (0–3.5mM) which gave $\lambda_{max.}$ 610nm and $\varepsilon = 16330$ litre·mol^{-1}·cm^{-1} for the tryptophan-4-dimethylaminobenzaldehyde adduct. A sample of *N*-acetyl-DL-tryptophan (1.4mM) in 0.05M-acetate buffer, pH5.0 treated with *N*-bromosuccinimide (molar ratio *N*-bromosuccinimide/*N*-acetyl-DL-tryptophan = 1.5) was subjected to this tryptophan-estimation procedure. No absorption at 610nm was detected. It was also shown that *N*-bromosuccinimide-oxidized tryptophan does not interfere with the estimation of unmodified tryptophan.

Determination of the thiol content of papain. Mercuri-papain or papain-SS-C_2H_4OH solution (2ml, approx. 50μM) in sodium phosphate buffer (0.1M; pH8.0; 1mM-EDTA) was activated by the addition of 2-mercaptoethanol (2μl). After 15min the papain was separated from activator by gel filtration on a column (13cm×1.5cm) of Sephadex G-25 (fine grade) in the same phosphate buffer and fractions (1.2ml) were collected. A portion (1.0ml) of one of the fractions containing papain was placed in a 1cm path-length cuvette and the absorbance at 280nm recorded. 5,5′-Dithiobis-(2-nitrobenzoic acid) (50μl; 10mM in the phosphate buffer, pH8.0) was added and the increase in absorbance at 412nm was noted after 6min. This was repeated for the other papain-containing fractions and for a 1.0ml buffer sample to obtain a blank reading. A value of $\varepsilon = 13600$ litre·mol^{-1}·cm^{-1} per thiol group at 412nm (Ellman, 1959) was used to calculate the molar ratio of thiol/papain for each fraction. The thiol content before peptide 'mapping' was measured from the radioactivity incorporated after reaction with iodo[^{14}C]-acetate.

Preparative paper chromatography. Peptide solutions were chromatographed on Whatman 3MM paper with *n*-butanol–acetic acid–water (40:6:15, by vol.). Development of guide strips with Ehrlich's reagent (Dawson *et al.*, 1969*b*) identified those peptides containing tryptophan, which were cut out and eluted with water.

Preparative paper electrophoresis. High-voltage (3kV) paper electrophoresis of the peptide solutions was performed on Whatman 3MM paper at pH6.5 [pyridine (500ml) and acetic acid (15ml) made up to 5 litres with water]. The tryptophan-containing peptides were located as before, and the paper bearing them was cut out and sewn on to a separate strip of Whatman 3MM paper. After electrophoresis at pH3.5 [pyridine (16.6ml) and acetic acid (166ml) made up to 5 litres with water] the peptides were located as before and eluted from the paper with water.

Peptide mapping. The freeze-dried α-chymotryptic digest of reduced carboxymethylated iodo[^{14}C]-acetate-inhibited papain (5mg) was suspended in water (0.25ml) and, after centrifugation, the clear supernatant was applied as a 2cm streak near the centre of a sheet of Whatman 3MM paper. A spot of Xylene Cyanol FF was applied at each edge of the paper and electrophoresis at pH6.5 was continued until the dye had moved 10cm. The paper was dried, the marker dye cut off and an 11cm strip was removed from the cathode end. The paper was sewn on to a new sheet of Whatman 3MM paper and electrophoresis at pH3.5 performed perpendicular to the direction of the electrophoresis at pH6.5 until the marker dye applied to the edges had moved 18cm. The radioactive peptides were located by radioautography and the tryptophan-containing peptides were located with Ehrlich's reagent.

Spectroscopic method of measuring kinetic parameters. Mercuri-papain or papain-SS-C_2H_4OH solution (10–25μl; approx. 10μM) was activated with cysteine or 2-mercaptoethanol (10mM) and EDTA (1mM) at pH5–7 for at least 20min. The activated enzyme was added to the assay buffer (2.5ml) in a 1cm path-length cuvette in the thermostatically controlled cell compartment of a Unicam SP. 1800 spectrophotometer. The mixture was left for 20min to reach thermal equilibrium and then *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester in acetonitrile (25μl) was added, mixed quickly and the change in absorbance at 340nm recorded. In some cases it was convenient to activate the papain *in situ* by using an assay buffer containing cysteine (1mM). The 20min thermal-equilibrium period was sufficient for complete activation of the papain. A blank run without enzyme was performed to correct for non-enzymic rate of hydrolysis.

To obtain the Michaelis–Menten parameters the initial rate of papain-catalysed hydrolysis was measured at several substrate concentrations and the data evaluated by the method of Wilkinson (1961).

When the total-progress method was used the papain-catalysed hydrolysis was followed to completion and automatically recorded with a Stogate data logger (model SDS/TP) connected to the Unicam SP. 1800. At regular time-intervals, the data logger measured a voltage directly proportional to the absorbance at 340nm and recorded it on punched paper tape. A few minutes of a blank run were likewise recorded and the Michaelis–Menten parameters computed by using a program that fits experimental points of the total-progress curve to a third-order polynomial, differentiates the polynomial to give the reaction velocity at different concentrations of substrate, corrects the reaction velocity for blank rate and treats this data by the method of Wilkinson (1961).

Measurement of kinetic parameters with a pH-stat
This method was used for the substrates *N*-benzyloxycarbonylglycylglycine amide and *N*-benzyloxycarbonylglycyl-L-tryptophan amide. The reaction conditions are summarized in Table 4.

The initial rate of hydrolysis, V, (less than 5% hydrolysis, giving linear slopes) was measured at several substrate concentrations, [S], and the Michaelis–Menten parameters computed by using the method of Wilkinson (1961). The ratio $k_{cat.}/K_m$ was calculated from the ratio $V_{max.}/K_m$, which was obtained from a linear least-squares fit to a plot of $[S]/V$ against [S].

Other methods

Preparation of mercuri-papain. Twice-crystallized papain, prepared by the method of Kimmel & Smith (1954), was purified by affinity chromatography on an agarose–mercurial column prepared by the method of Sluyterman & Wijdenes (1970).

Preparation of active papain. Twice-crystallized papain was purified by affinity chromatography by the method of Blumberg *et al.* (1970) by using a column (25cm×2.3cm) of glycylglycyl-(*O*-benzyl)-L-tyrosyl-L-arginine covalently linked to Sepharose 4B.

2-Mercaptoethanol (0.2ml) was added to a solution of papain (0.75g) in 20mM-EDTA buffer (100ml, pH4.5) and kept for 20min before the mixture was applied to the column that was already equilibrated with buffer. Inactive papain was completely removed from the column by elution with buffer before the active papain was eluted with water. The papain was assayed with *N*-α-benzoyl-L-arginine ethyl ester at pH6.0 and 25°C, and, assuming $K_m = 18$mM, $k_{cat.}$ was found to be $27.7s^{-1}$, in agreement with the values found by Sluyterman & Wijdenes (1970) ($k_{cat.} = 26s^{-1}$) and by Blumberg *et al.* (1970) ($k_{cat.} = 28.5s^{-1}$).

Preparation of papain-SS-C_2H_4OH. To a solution of active papain (42ml; 92μM) was added EDTA (disodium salt; 15.6mg) and 2-hydroxyethyl disulphide (25μl), giving a solution of pH4.5. The mixture was kept at 4°C for 3 days, and assay with *N*-α-benzoyl-L-arginine ethyl ester in the absence of cysteine (i.e. without activation) showed 6.4% of active enzyme remaining. In the presence of 5mM-cysteine the hydrolysis rate increased over 20min to a maximum value of 94% of the original activity.

The residual 6.4% of active papain was removed by applying the solution to a column (5cm×1.5cm) of agarose–mercurial and eluting with water. Assay of papain-SS-C_2H_4OH in the eluate with *N*-α-benzoyl-L-arginine ethyl ester in the absence of cysteine showed zero activity. Ultrafiltration with water gave papain-SS-C_2H_4OH solution (22ml; 118μM) containing 2-hydroxyethyl disulphide (approx. 80μM).

Inhibition of papain with dibromoacetone. Papain-SS-C_2H_4OH (2.5ml) 0.116mM) in sodium acetate buffer (50mM; pH5.1) containing EDTA (1mM) was activated by the addition of 2-mercaptoethanol (10μl) and after 30min was eluted on a column (20cm×1.5cm) of Sephadex G-25 (fine grade) in the same buffer. The papain-containing fractions were combined, 1,3-dibromoacetone (Husain & Lowe, 1968) (1mg in 0.5ml of acetone) was added and after stirring for 10min no activity was detectable on assay with *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester. The inhibited papain solution was concentrated to 2.5ml, separated from excess of inhibitor by elution on the Sephadex G-25 column as above and concentrated to approx. 2ml (75nM). Amino acid analysis showed 0.95 residues of histidine (normalized to 12 arginine residues).

Effect of N-bromosuccinimide on mercuri-papain. Solutions of mercuri-papain (each 2.4ml; 30.5μM) in sodium acetate buffer (50mM, pH4.5) were treated, while being stirred vigorously, with 0, 2, 4, 6, 8 and 10 molar equiv. of *N*-bromosuccinimide by the addition of 0, 25, 50, 75, 100 and 125μl of aqueous *N*-bromosuccinimide solution (5.84mM) respectively.

Preparation of N-bromosuccinimide-modified papain SS-C_2H_4OH. Nine papain-SS-C_2H_4OH solutions (5ml each; 120μM) in sodium acetate buffer (50mM; pH4.5) were treated with 0, 1, 2, 3, 4, 6, 9, 14 and 20 molar equiv. of *N*-bromosuccinimide respectively. The aqueous *N*-bromosuccinimide solution (25–200μl of a suitable concentration) was added slowly from a micropipette to the vigorously stirred papain solution at room temperature. The solutions of *N*-bromosuccinimide-modified papain-SS-C_2H_4OH were stored at 2–4°C until required.

Ion-exchange chromatography of N-bromosuccinimide (4 molar equiv.)-modified papain-SS-C_2H_4OH. A preliminary small-scale investigation of 4-*N*-bromosuccinimide–papain-SS-C_2H_4OH on Bio-Rex 70 (Bio-Rad Laboratories, St. Albans, Herts., U.K.). led to a pH6.50 buffer containing 0.08M-phosphate and 0.16M-Na^+ being selected for the purification of the modified enzyme (see Fig. 2).

Active-site labelling of papain with iodo[^{14}C]-acetate. Papain-SS-C_2H_4OH (5ml; approx. 90μM) in sodium acetate buffer (80mM; pH5.0) was activated by the addition of 2-mercaptoethanol (5μl). The mixture was kept for 30min at room temperature, iodo[^{14}C]acetate (0.2ml; 5.5mM; 4.5Ci/mol) in dimethyl sulphoxide was added with stirring and the solution kept in the dark for 2h. Assay of the solution with *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester showed less than 0.2% activity remaining. The ^{14}C-labelled protein was separated from excess of inhibitor by gel filtration on a column (30cm×2cm) of Sephadex G-25 (fine grade) in 0.1M-acetic acid and freeze-dried after taking a sample for scintillation counting of radioactivity.

By the same method, samples of 4-*N*-bromosuccinimide–papain-SS-C_2H_4OH from both peak I and II were inhibited with iodo[^{14}C]acetate and the extent of incorporation of the inhibitor was determined by scintillation counting. 2-*N*-Bromosuccinimide–papain-SS-C_2H_4OH, carboxymethylated at cysteine-25, was also prepared by a similar procedure with unlabelled iodoacetic acid.

Reduction and carboxymethylation of papain. Iodoacetic acid (2.76mg; 14.9μmol) dissolved in water (1ml) was added slowly to a stirred solution of active papain (313mg; 13.5μmol) in sodium acetate buffer (300ml; 10mM; pH4.9) containing 1mM-EDTA. After the mixture had been kept overnight at 4°C, less than 0.05% of activity remained. The buffer concentration was decreased to approx. 1mM by ultrafiltration and dilution and the solution was freeze-dried.

The freeze-dried carboxymethylated papain was dissolved in a solution of tris(hydroxymethyl)aminomethane (5.5g), guanidinium chloride (15g) and EDTA (47mg) in de-gassed water (15ml). The solution was adjusted to pH8.6 by the addition of 12M-HCl (approx. 2ml) and incubated with 2-mercaptoethanol (0.4ml) at 35°C under N_2 for 5h.

Iodoacetic acid (1.07g) in 6M-guanidinium chloride solution [adjusted to pH8.6 by the addition of solid tris(hydroxymethyl)aminomethane] was added with stirring and the mixture kept for 30min in the dark under N_2. 2-Mercaptoethanol (1ml) was added to react with excess of iodoacetate and the solution was dialysed against water (4×5 litres) in the dark for 30h.

Amino acid analysis of a sample of the dialysed carboxymethylated papain showed 7.26 residues of *S*-carboxymethylcysteine (normalized to 14 residues of alanine).

Digestion with α-chymotrypsin. The aqueous suspension of reduced carboxymethylated protein after dialysis was digested with α-chymotrypsin (6mg) at 32°C in a pH-stat under N_2 and the pH was kept at 8.0 by the automatic addition of 0.1M-NaOH. After 12h the digestion had stopped and did not recommence on the addition of more α-chymotrypsin (1mg). The digest was freeze-dried immediately and stored at 4°C.

Isolation of tryptophan-containing peptides from the α-chymotryptic digest of papain. The freeze-dried chymotryptic digest of reduced carboxymethylated papain was suspended in water (2ml), centrifuged and the supernatant filtered through a Millipore filter before application to a column of Sephadex G-25 (fine grade) (see Fig. 4).

After preliminary chromatography of selected fractions (between 93 and 133) on Whatman no. 1 paper in *n*-butanol–acetic acid–water (40:6:15, by vol.), fractions 93–100, 102–107 and 113–133 were pooled and subjected to preparative paper chromato-

graphy and electrophoresis (solvents and pH as above). After isolation of the pure peptides, amino acid analysis allowed their identity to be established by comparison with the known sequence of papain (Husain & Lowe, 1969). From their mobilities at pH3.5 and 6.5 the spots in Fig. 3 could be identified (Table 2).

Results and Discussion

The protection of the active-site thiol group of papain was clearly a prerequisite to the modification of tryptophan residues with *N*-bromosuccinimide. Since, however, the activity of the modified enzyme was one of the properties of interest it was necessary to achieve the protection in a readily reversible manner. Treatment of mercuri-papain (Sluyterman & Wijdenes, 1970) with *N*-bromosuccinimide undoubtedly modified some of the tryptophan residues as indicated by the change in the u.v. absorption spectrum, but it was evident from the thiol content (0.11 residues/mol of papain with 6 molar equiv. of *N*-bromosuccinimide) that the mercury was not adequately protecting the thiol group.

Fully active papain was therefore prepared from the twice-crystallized enzyme by affinity chromatography on a column of Sepharose 4B to which the tetrapeptide glycylglycyl-(*O*-benzyl)tyrosylarginine had been coupled (Blumberg *et al.*, 1970). The active enzyme after incubation for 3 days at 2–4°C with 2-hydroxyethyl disulphide had lost almost all enzymic activity, but could be reactivated by incubation with cysteine. However, some irreversible inactivation occurred during this process since only 94% enzymic activity was recovered. When 0.93±0.02 thiol residues/mol of enzyme were found with Ellman's reagent it was clear that the loss of activity was due to oxidation of the thiol group. This irreversibly inactivated component was assumed to behave in the same way as the activatable enzyme (papain-SS-C_2H_4OH) towards *N*-bromosuccinimide and the kinetic parameters (in Table 1) for the variously modified enzyme preparations are not corrected for the presence of this small amount of inactive protein. Oxidation of the protected enzyme with 6 molar equiv. of *N*-bromosuccinimide did not result in any loss of thiol content after reactivation. This form of the protected enzyme was used therefore for all subsequent oxidation studies with *N*-bromosuccinimide.

As shown in Table 1, papain-SS-C_2H_4OH was treated with *N*-bromosuccinimide at a series of molar proportions. The change in the u.v.-absorption spectrum (Fig. 1) is indicative of oxidation of tryptophan residues to the oxindole or bromo-oxindole derivatives. Although the change in u.v. absorption after oxidation with *N*-bromosuccinimide has often been used to estimate the number of tryptophan residues oxidized, discrepancies have been known to arise between the results of this and other methods of estimating tryptophan oxidation (Kronman *et al.*, 1967). In the present work a procedure for tryptophan assay adapted from that of Graham *et al.* (1947) was used.

The amino acids that may be affected by *N*-bromosuccinimide are cysteine, methionine, tryptophan, tyrosine and histidine (Perham, 1969), but as it has already been shown that the single cysteine in papain is adequately protected against this

Table 1. *Properties of papain protected as a disulphide after modification with N-bromosuccinimide*

Papain-SS-C_2H_4OH solutions modified by different molar equivalents of *N*-bromosuccinimide at pH4.5 were assayed colorimetrically for tryptophan content, by amino acid analysis for histidine (relative to arginine, 12 residues) and tyrosine (relative to alanine, 14 residues), for fluorescence intensity (concentration 1.2μM) by observing the emission at 340nm on excitation at 288nm in McIlvaine's phosphate–citrate buffer, pH6.90, containing 0.3M-Na^+ and 1mM-EDTA, and for the Michaelis parameters with *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester at 35°C and pH5.0 in 0.1M-acetate–0.3M-NaCl–1mM-EDTA–1mM-cysteine containing 1% (v/v) acetonitrile by using the total-progress method and selecting ten points from each curve.

Molar ratio of *N*-bromosuccinimide/ papain	Molar ratio of Trp/ papain	Molar ratio of His/ papain	Molar ratio of Tyr/ papain	Relative fluorescence intensity	K_m (μM)	$k_{cat.}$ (s^{-1})	$10^{-6}\times k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)
0	5.07±0.08	1.93±0.02	18.48±0.17	100	6.68±0.10	11.06±0.05	1.64
1	4.40±0.03	1.92±0.01	18.75±0.05	95.6	5.54±0.06	9.25±0.02	1.66
2	4.03±0.08	1.93±0.03	18.51±0.16	92.0	3.60±0.05	7.98±0.02	2.20
3	3.50±0.07	1.96±0.01	18.76±0.24	71.9	3.29±0.05	6.82±0.02	2.07
4	2.96±0.06	1.90±0.08	18.41±0.01	45.5	2.78±0.05	4.48±0.01	1.61
6	2.73±0.11	1.86±0.05	17.28±0.41	40.9	2.61±0.06	3.73±0.01	1.42
9	2.49±0.05	1.85±0.04	16.18±0.03	33.1	2.74±0.04	3.02±0.01	1.10
14	2.36±0.04	1.77±0.03	14.55±0.13	21.4	3.37±0.05	2.52±0.01	0.75
20	2.04±0.03	1.62±0.05	11.90±0.10	14.9	4.89±0.07	1.68±0.01	0.34

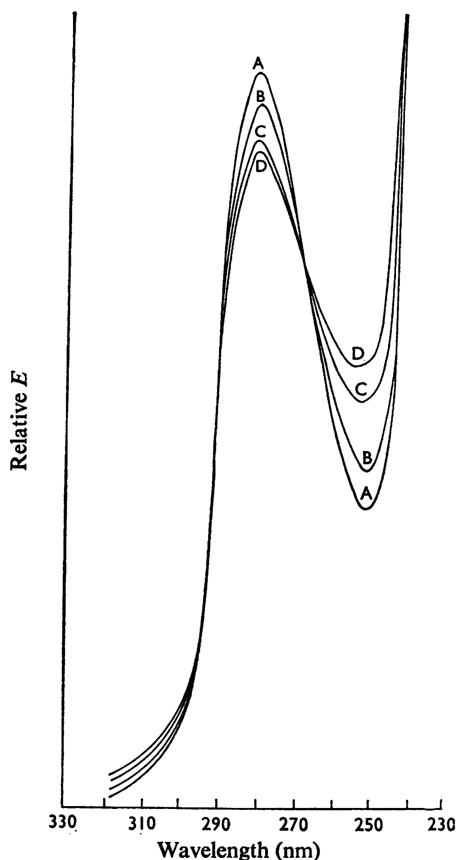

Fig. 1. *Effect of N-bromosuccinimide on the u.v. spectrum of papain-SS-C_2H_4OH (24.6μM) at pH4.5 in sodium acetate buffer (50mM)*

The molar ratio of *N*-bromosuccinimide to papain-SS-C_2H_4OH is: curve A, 0.0; curve B, 3.0; curve C, 6.0; curve D, 9.0.

reagent by disulphide formation with 2-mercaptoethanol and as methionine is absent from papain, only analyses for tryptophan, tyrosine and histidine were carried out.

The amino acid analysis of papain-SS-C_2H_4OH treated at pH4.5 with up to 4 molar equiv. of *N*-bromosuccinimide showed no change in the histidine or tyrosine content whereas the tryptophan content dropped from 5 to 3 residues/papain molecule (Table 1). Further addition of *N*-bromosuccinimide up to a total of 20 molar equiv. caused a progressive loss of about 7 tyrosine residues accompanied by the steady loss of 1 more tryptophan residue and about one-third of a histidine residue.

The data also indicate that the equivalent of 1 tryptophan residue is oxidized by 2 molar equiv. of

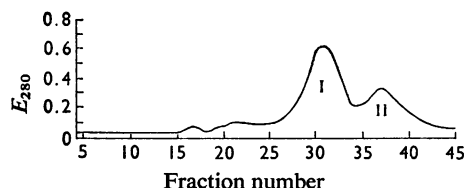

Fig. 2. *Gel filtration of 4-N-bromosuccinimide–papain-SS-C_2H_4OH (20ml, 0.15mM) in 0.08M-phosphate buffer, pH6.50, on a column (200cm × 2cm) of Bio-Rex 70*

The column was eluted at 44ml/h and 22ml fractions were collected. Fractions 30 and 36 were used to represent peaks I and II for kinetic and fluorimetric studies. For peptide 'mapping' and amino acid analysis fractions 27–32 were used from peak I and fractions 35–45 from peak II.

N-bromosuccinimide. The fluorescence spectra of papain-SS-C_2H_4OH modified by 2 molar equiv. and 4 molar equiv. of *N*-bromosuccinimide show a fall in intensity relative to that of the active enzyme of 8% and 54.5% respectively, clearly indicating that the 2 susceptible tryptophan residues are selectively oxidized, the first to be oxidized contributing only about 8% to the overall fluorescence intensity of the native enzyme and the second residue contributing almost one-half of the intensity. It has previously been observed by fluorescence-quenching experiments that 1 of the 5 tryptophan residues contributes about one-half of the fluorescence intensity (Steiner, 1971), but the identity of this residue has not so far been rigorously established. The kinetic parameters (see Table 1) obtained for *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester show that only modest changes occur in the enzymic activity even after extensive oxidation, provided the active-site thiol group is adequately protected and that when the two most susceptible tryptophan residues have been oxidized (with 4mol of *N*-bromosuccinimide) the lower catalytic constant ($k_{cat.}$) is just about compensated for by the Michaelis constant (K_m), so that the overall enzymic activity ($k_{cat.}/K_m$) is essentially unaffected. Thus although intrinsic binding energy of the modified enzyme for this substrate is not changed, some of the advantage of the use of binding energy to facilitate catalysis has been lost (Lowe & Yuthavong, 1971).

To be able to interpret the properties of the modified forms of papain in terms of its tertiary structure it is necessary to establish which of the tryptophan residues are oxidized. Before undertaking this structural investigation, however, papain-SS-C_2H_4OH, which had been modified by 4 molar equiv. of *N*-bromosuccinimide, was chromatographed on Bio-Rex 70 (Fig. 2). Two main com-

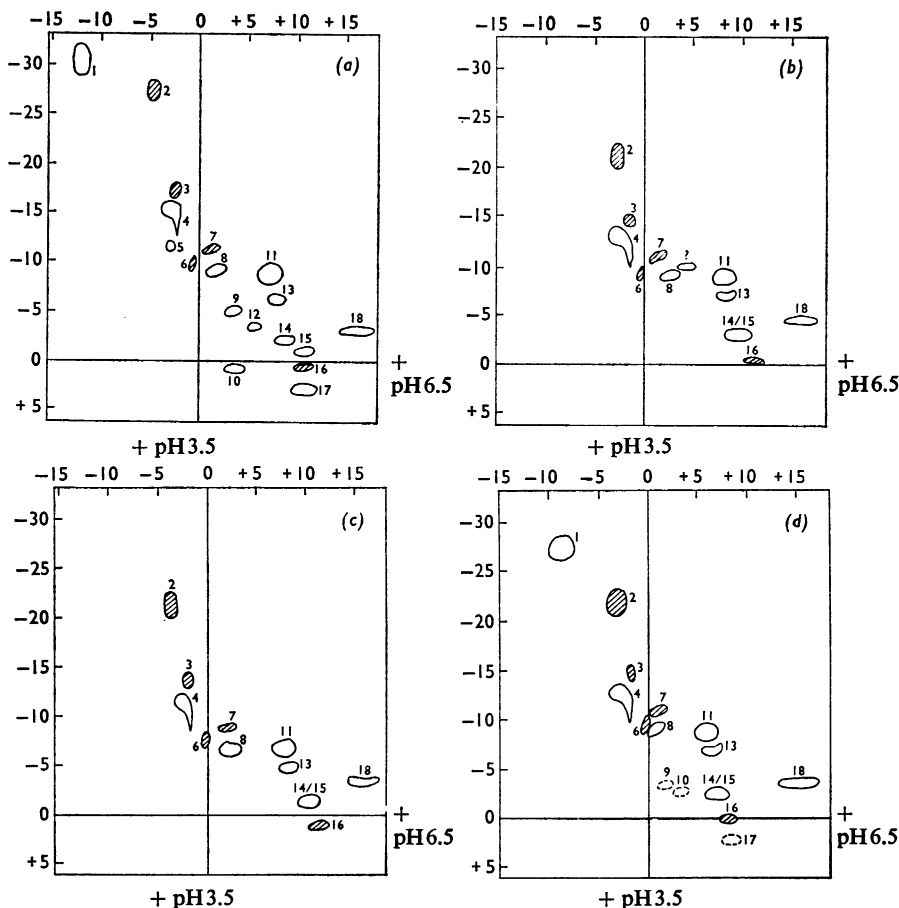

Fig. 3. *Peptide 'maps' (the axes being in cm) of the α-chymotryptic digest of reduced carboxymethylated iodo[^{14}C]acetate-inhibited enzyme: (a) native papain; (b) 4-N-bromosuccinimide-papain-SS-C_2H_4OH, peak I; (c) 4-N-bromosuccinimide-papain-SS-C_2H_4OH, peak II; (d) 2-N-bromosuccinimide-papain-SS-C_2H_4OH*

Radioactive spots are shaded and are assumed to contain tryptophan-26 adjacent to ^{14}C-labelled carboxymethylcysteine. The other spots are all peptides containing tryptophan and appeared on developing the paper with Ehrlich's reagent. All electrophoreses were run until marker dye Xylene Cyanol FF had run 10cm at pH6.5 and 18cm at pH3.5. The potential gradient was 55V/cm. At pH6.5 electrophoresis took 50–60min and at pH3.5, 80–120min.

ponents were resolved, and the protein in both peaks I and II contained 3 tryptophan residues to 1 reactivatable thiol group. Since the same 2 tryptophan residues have been modified in the protein of peaks I and II (see below), the observation suggests that the degree of modification is different or that the native enzyme is a mixture of two isoenzymes whose chromatographic differences are enhanced after *N*-bromosuccinimide modification.

Identification of the tryptophan residues modified by N-bromosuccinimide

Papain-SS-C_2H_4OH was activated and irreversibly inhibited with iodo[$^{14}C_2$]acetic acid to label radioactively the thiol of cysteine-25. It was reduced and carboxymethylated, digested with α-chymotrypsin and a two-dimensional peptide 'map' of the digest prepared. After radioautography, the 'map' was developed with Ehrlich's reagent.

The radioactively labelled cysteine-25 is adjacent to tryptophan-26 in the amino acid sequence of papain and, as the peptide bond between these two residues is unlikely to be cleaved by α-chymotrypsin, all the radioactive peptides will contain tryptophan-26. Hence the radioautograph identified spots, 2, 3, 6, 7 and 16 (see Fig. 3*a*) on the 'map' of papain-SS-C_2H_4OH as peptides containing tryptophan-26.

Tryptophan-containing peptides were isolated from the α-chymotryptic digest of reduced carboxy-

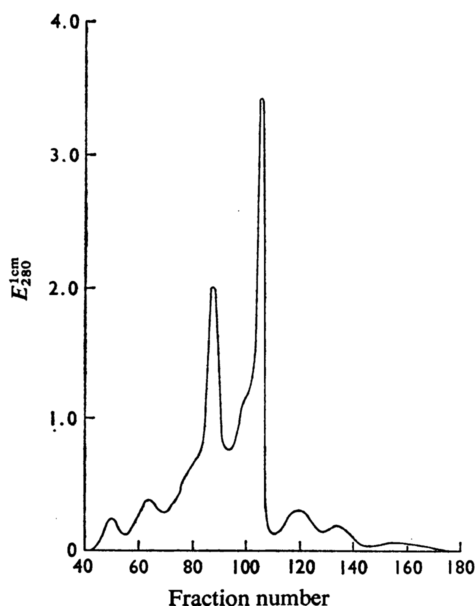

Fig. 4. *Gel filtration of the α-chymotryptic digest of reduced carboxymethylated papain on a column* (140 cm × 1 cm) *of Sephadex G-25 made up with deionized water*

The column was eluted at 11 ml/h and 1.1 ml fractions were collected.

methylated papain by the method of Husain & Lowe (1970) (Fig. 4) and purified by two-dimensional paper electrophoresis under the same conditions used for peptide 'mapping'. The amino acid analysis of each peptide enabled its position in the primary sequence of papain to be determined and identification of the peptides with the relevant spots on the peptide 'map' were made by comparison of their two-dimensional electrophoretic mobilities (see Table 2).

The peptide 'maps' prepared from 4-*N*-bromosuccinimide–papain-SS-C_2H_4OH from peaks I and II (Fig. 3*b* and 3*c*) did not contain spots 1, 9, 10, 12 and 17, which identified the 2 oxidized residues as tryptophan-69 and -177. The other 3 tryptophan residues had been unaffected, since all the radioactive spots showed up with Ehrlich's reagent, confirming that tryptophan-26 was unoxidized, and spots 4, 8, 13 and 18 were present, confirming that tryptophan-181 and -7 were also unoxidized.

In the peptide 'map' prepared from 2-*N*-bromosuccinimide–papain-SS-C_2H_4OH (Fig. 3*d*), spots 9, 10, 12 and 17 (due to tryptophan-69) were only weakly evident or absent, whereas spot 1 (due to tryptophan-177) was present at about its normal strength. Thus it would appear that the residue oxidized in 2-*N*-bromosuccinimide–papain-SS-C_2H_4-OH is essentially tryptophan-69.

The finding that the tryptophan residues preferentially modified by *N*-bromosuccinimide are residues

Table 2. *Identification of the spots on the peptide 'map' of unmodified papain*

The spot numbers correspond to those shown in Fig. 3(*a*). Mobilities are in cm travelled towards the anode; all electrophoreses were run until the marker dye Xylene Cyanol FF had run 10 cm at pH 6.5 and 18 cm at pH 3.5. The authentic peptides were obtained by gel filtration (see Fig. 4) and purified by paper chromatography and paper electrophoresis. Amino acid analysis allowed the sequences to be deduced from the amino acid sequence of the enzyme (Husain & Lowe, 1969; Mitchel *et al.*, 1970) and hence the tryptophan residue in each spot to be identified. The radioactive spots were assumed to contain tryptophan-26 since the enzyme had been labelled with iodo[$^{14}C_2$]acetic acid at cysteine-25. CmCys, carboxymethylcysteine. For further details see the text.

Spot number	Mobility (cm) pH 6.5	Mobility (cm) pH 3.5	Mobility (cm) of authentic peptide pH 6.5	Mobility (cm) of authentic peptide pH 3.5	Amino acid sequence	Tryptophan residue
1	−11.5	−30	−12.3	−29	Ile-Lys-Asn-Ser-Trp	177
2						26
3						26
4	−4	−15	−4.5	−16	Gly-Thr-Gly-Trp	181
6						26
7						26
8	+2	−9	+3	−10	Gly-Thr-Gly-Trp-Gly-Glu-Asn-Gly-Tyr	181
9	+3	−4.7	+3.2	−5	Gly-Cys-Asp-Gly-Gly-Tyr-Pro-Trp	69
10	+3.5	+0.5	+3	+0.5	Same analysis as no. 9	69
12	+6	−3.5	+5	−3	Same analysis as no. 9	69
13	+8	−6	+9	−7	Gly-Thr-Gly-Trp-Gly-Glu-Asn-Gly-Tyr	181
16						26
17	+10.5	+3	+11	+3	Gly-CmCys-Asp-Gly-Gly-Tyr-Pro-Trp	69
18	+16	−3.7	+17	−4.5	Ile-Pro-Glu-Tyr-Val-Asp-Trp	7

69 and 177 is not unexpected since these are located on the surface of the protein and well exposed to the solvent, whereas the other 3 tryptophan residues are either partially or completely buried.

The preferential attack of *N*-bromosuccinimide on tryptophan-69 could be due to its slightly greater degree of exposure to the solvent compared with that of tryptophan-177, but this may not be the only factor involved. As tryptophan-69 is one of the residues lining a well defined hydrophobic pocket in the enzyme, even weak hydrophobic binding of *N*-bromosuccinimide would tend to direct the reagent to the vicinity of this tryptophan residue and hence aid its preferential modification.

Fluorescence of N-bromosuccinimide-modified papain-SS-C_2H_4OH

As the addition of the 2 molar equiv. of *N*-bromosuccinimide causes essentially the oxidation of only tryptophan-69, it would appear from the observed 8% fall in fluorescence intensity (Table 1) that this tryptophan residue has a relatively low quantum yield and does not contribute greatly to the total fluorescence spectrum of papain. The much greater loss of fluorescence intensity (46%) on the addition of another 2 molar equiv. of *N*-bromosuccinimide must be due to the loss of tryptophan-177, suggesting that this residue has a relatively high quantum yield and is responsible for almost one-half of the fluorescence intensity of papain at neutral pH.

The effects of pH on the fluorescence of the papain species modified with *N*-bromosuccinimide are shown in Fig. 5. For comparison papain-SS-C_2H_4OH was also studied and showed quenching of tryptophan fluorescence both at acidic pH, with an apparent pK_a of 4.2, and at alkaline pH, in close agreement with the work of Sluyterman & de Graaf (1970). The fluorescence of 2-*N*-bromosuccinimide–papain-SS-C_2H_4-OH still showed a pH profile with apparent pK_a 4.2, but with about 8–10% loss of fluorescence intensity at neutral pH, whereas 4-*N*-bromosuccinimide–papain-SS-C_2H_4OH, from either peak I or peak II, showed no variation of fluorescence with pH in the acid region and the fluorescence intensity was lowered to about the same value as that of acid-quenched unmodified papain-SS-C_2H_4OH.

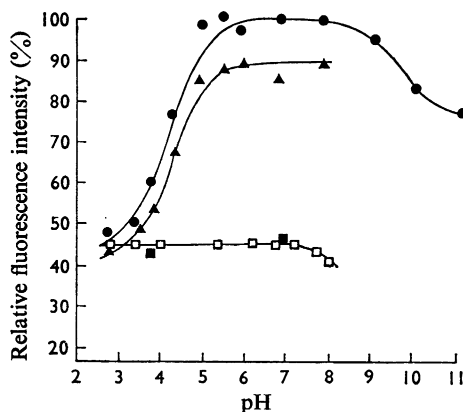

Fig. 5. *Effect of pH on the fluorescence of papain-SS-C_2H_4OH (●), 2-N-bromosuccinimide–papain-SS-C_2H_4OH (▲), and 4-N-bromosuccinimide–papain-SS-C_2H_4OH, peak I (□) and peak II (■)*

The lines drawn through the data for unmodified and 2-*N*-bromosuccinimide–papain-SS-C_2H_4OH are theoretical in the pH range 2–8 for a pK_a of 4.2. Papain concentration was approx. 1.2μM in McIlvaine's phosphate–citrate buffer containing, 0.3M-Na^+ and the excitation and emission wavelengths were 288nm and 340nm respectively. The fluorescence intensity at the $\lambda_{max.}$ was expressed relative to that of papain-SS-C_2H_4OH at pH6.90, with corrections for slight differences in concentration.

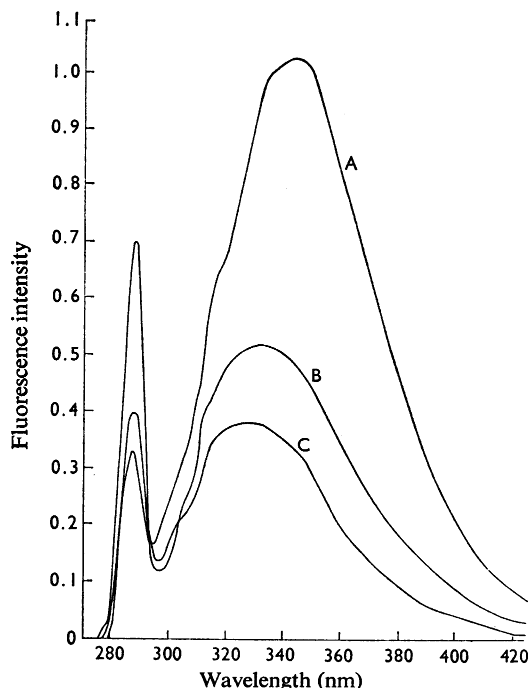

Fig. 6. *Fluorescence spectra of papain species, excited at 288nm, dissolved in McIlvaine's phosphate–citrate buffer containing 0.3M-Na^+ and 1mM-EDTA*

Curve A, native papain-SS-C_2H_4OH, 1.44μM, pH6.9; curve B, native papain-SS-C_2H_4OH, 1.44μM, pH3.45; curve C, 4-*N*-bromosuccinimide–papain-SS-C_2H_4OH, 1.16μM, pH6.9.

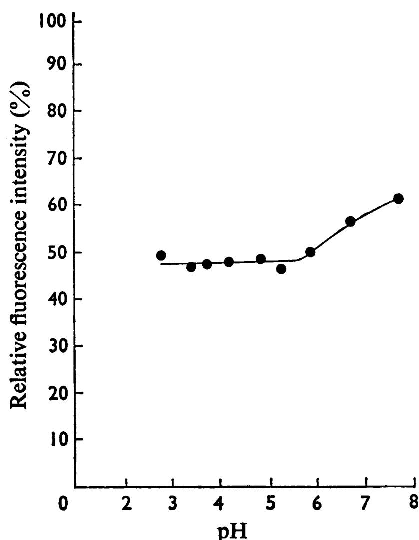

Fig. 7. *pH-dependence of the fluorescence intensity of 1,3-dibromoacetone-inhibited papain relative to the fluorescence intensity of papain-SS-C_2H_4OH under the same experimental conditions as in Fig.* 5

Since both unmodified and 2-*N*-bromosuccinimide-papain-SS-C_2H_4OH contain tryptophan-177 essentially intact and showed pH-dependence of fluorescence intensity, whereas 4-*N*-bromosuccinimide–papain-SS-C_2H_4OH, in which tryptophan-177 is completely oxidized, showed no pH-dependence, it is evident that it is the fluorescence of this tryptophan residue that is quenched at acid pH and is responsible for about one-half of the total fluorescence of the protein at neutral pH.

The relative intensity of fluorescence of 4-*N*-bromo-succinimide–papain-SS-C_2H_4OH is about the same as that of unmodified papain-SS-C_2H_4OH at low pH (Fig. 5), and their fluorescence spectra (Fig. 6, curves B and C) show almost identical $\lambda_{max.}$ values. These two species give similar fluorescence spectra under these conditions, the spectral contribution from tryptophan-69 to the unmodified papain-SS-C_2H_4OH accounting for the difference.

Speculation by Steiner (1971) that tryptophan-26 might be the dominant tryptophan residue and the tentative conclusion by Sluyterman & Wijdenes (1972) that the pH profile of fluorescence of papain is due to quenching the fluorescence of tryptophan-26 by the undissociated aspartic acid-57 are both disproved, as this tryptophan residue has been shown to be unmodified in 4-*N*-bromosuccinimide–papain-SS-C_2H_4OH. By far the closest ionizable group to the indole ring of tryptophan-177 is the imidazole of histidine-159, and the pH-dependent quenching of the fluorescence spectrum can most reasonably be associated with this group.

Papain-SS-C_2H_4OH was activated and inhibited with 1,3-dibromoacetone at pH 5.1 to cross-link histidine-159 with cysteine-25 (Husain & Lowe, 1968). An enzymic assay showed that total inhibition had occurred and amino acid analysis showed a loss of 1.0 histidine residue. The fluorescence intensity of this papain species (Fig. 7) is considerably lower than that of papain-SS-C_2H_4OH even at neutral pH and is less sensitive to changes in pH. It would appear therefore that the alkylated imidazole of histidine-159 disturbs the micro-environment of tryptophan-177. Although the exact nature of this quenching is unclear, it is apparent that the fluorescence contribution of tryptophan-177 is drastically affected by modification of histidine-169.

Catalytic activity of N-bromosuccinimide-modified papain

The pH-dependence of the hydrolysis of *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester catalysed by 2-*N*-bromosuccinimide–papain-SS-C_2H_4OH and 4-*N*-bromosuccinimide–papain-SS-C_2H_4OH from both peaks I and II (after activation) was investigated. The apparent pK_a values are summarized in Table 3 and compared with those of native papain. For this substrate $k_{cat.}$ represents the deacylation rate constant, k_3, and $k_{cat.}/K_m$ represents k_2/K_s. Assuming K_s is constant in the pH range under investigation, the pH-dependence of $k_{cat.}/K_m$ represents the pH-dependence of the acylation rate constant, k_2.

Table 3 shows that native papain and 2-*N*-bromo-succinimide–papain-SS-C_2H_4OH (after activation) have almost the same apparent pK_a value for $k_{cat.}$ and the apparent pK_a values for $k_{cat.}/K_m$ differ by only 0.4 p*K* unit. This suggests that the oxidation of tryptophan-69 has practically no effect on the pH-dependence of deacylation, but causes a slight elevation of the pK_a of acylation. For 4-*N*-bromo-succinimide–papain-SS-C_2H_4OH (after activation) the apparent pK_a values derived from $k_{cat.}$ for the enzyme of peaks I and II are 1.05 and 0.75 p*K* units respectively above that for native papain, and the pK_a values derived from $k_{cat.}/K_m$ for enzyme of peaks I and II are 1.05 and 1.15 p*K* units respectively, higher than that for native enzyme. These marked increases in pK_a values are associated with the modification of tryptophan-177. Assuming that the acidic pK_a observed during substrate hydrolysis is that of the imidazole group of histidine-159, whose pK_a is unusually low owing to its hydrophobic micro-environment, the oxidation of tryptophan-177 would be expected to make it less hydrophobic and this should be reflected by an increase in the apparent

pK_a observed during substrate hydrolysis. Hence one role of tryptophan-177 in papain appears to be to contribute significantly to the lowering of the imidazole pK_a of histidine-159, enabling the enzyme to remain active over a larger pH range than would be the case if this histidine had a normal pK_a value.

The modified papain species were also studied with the substrates *N*-benzyloxycarbonylglycylglycine amide and *N*-benzyloxycarbonylglycyl-L-tryptophan amide to investigate any change in leaving-group specificity on modification with *N*-bromosuccinimide. The results are compared with the data for native papain in Table 4. The values of $k_{cat.}$ for these substrates are considerably smaller than that for *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester and so represent the acylation rate constant, k_2, and K_m represents K_s, the substrate dissociation constant. For *N*-benzyloxycarbonylglycylglycine amide, although the $k_{cat.}$ values for native and 4-*N*-bromosuccinimide–papain-SS-C_2H_4OH (after activation) are fairly similar, the K_m values differ by a factor of about six, representing weaker binding of the substrate by the *N*-bromosuccinimide-modified enzyme. With *N*-benzyloxycarbonylglycyl-L-tryptophan amide, oxidation of native protected enzyme to 2-*N*-bromosuccinimide–papain-SS-C_2H_4OH causes about a sixfold increase in K_m (after activation), $k_{cat.}$ remaining virtually unchanged, whereas oxidation to 4-*N*-bromosuccinimide–papain-SS-C_2H_4OH causes about an eightfold drop in $k_{cat.}$ (after activation) without further affecting K_m.

For *N*-benzyloxycarbonylglycyl-L-tryptophan amide, it appears that the oxidation of tryptophan-69 causes a decrease in substrate binding with little effect on the rate of acylation, whereas further oxidation (of tryptophan-177) to give 4-*N*-bromosuccinimide–papain-SS-C_2H_4OH causes no additional change in substrate binding, but a marked fall in the acylation rate constant. This suggests that these 2 tryptophan residues have independent roles. Oxidation of tryptophan-69 would be expected to decrease the hydrophobicity of the S_2 subsite and weaken binding of the *N*-benzyloxycarbonyl group, as is observed for both of these substrates. Oxidation of tryptophan-177 has little effect on the acylation rate constant with *N*-benzyloxycarbonylglycylglycine amide but has a marked effect with *N*-benzyloxycarbonylglycyl-L-tryptophan amide. This suggests that an interaction, which facilitates acylation, occurs between the tryptophan side chain of the substrate and tryptophan-177 on the enzyme and is impaired by oxidation of tryptophan-177. The similarity of $k_{cat.}$ for the native enzyme and 4-*N*-bromosuccinimide–papain-SS-C_2H_4OH (after activation) catalysed hydrolyses of *N*-benzyloxycarbonylglycylglycine amide, presumably is due to the complete absence of interaction with tryptophan-177, making the state of oxidation of this residue irrelevant. A possible mechanism by which this proposed interaction enhances acylation is that the interaction of the tryptophan amide-leaving group with the indole ring of tryptophan-177 increases the strain on the amide bond about to be cleaved, so that acylation is facilitated. When tryptophan-177 is oxidized interaction with the substrate would be diminished and the acylation rate decreased. Thus evidence is provided for tryptophan-177 forming a part of the S_1' subsite of papain.

Table 3. *Apparent* pK_a *values of* $k_{cat.}$ *and* $k_{cat.}/K_m$ *for the enzyme-catalysed hydrolysis of N-benzyloxycarbonylglycine p-nitrophenyl ester at* 35°C

For details see the text. The data for native papain are those of Yuthavong (1969).

	$k_{cat.}$	$k_{cat.}/K_m$
Native papain	3.50	4.30
2-*N*-Bromosuccinimide–papain-SS-C_2H_4OH	3.45	4.70
4-*N*-Bromosuccinimide–papain-SS-C_2H_4OH (peak I)	4.55	5.35
4-*N*-Bromosuccinimide–papain-SS-C_2H_4OH (peak II)	4.25	5.45

Table 4. *Hydrolysis of N-benzyloxycarbonylglycylglycine amide and N-benzyloxycarbonylglycyl-*L*-tryptophan amide by various papain species at pH*7.5 *and* 35°C *in* 20% (*v*/*v*) *dimethylformamide*–0.3M-*NaCl*–1mM-*EDTA*

The enzyme (2–30μM) was activated with mercaptoethanol (10mM). pK_a values of 7.52 and 7.90 for tryptophan amide and glycine amide were used to derive $k_{cat.}$ from the observed hydrolysis rate, and the data for native enzyme are from Alecio *et al.* (1974). At least five substrate concentrations were used for the determination of the parameters.

	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)
N-Benzyloxycarbonylglycylglycine amide			
Native papain	0.068±0.014	6.4±2.3	10.6
4-*N*-bromosuccinimide–papain-SS-C_2H_4OH	0.040±0.013	34±14	1.2
N-Benzyloxycarbonylglycyl-L-tryptophan amide			
Native papain	0.60±0.02	0.90±0.12	670
2-*N*-bromosuccinimide–papain-SS-C_2H_4OH	0.49±0.04	5.3±0.9	92
4-*N*-bromosuccinimide–papain-SS-C_2H_4OH	0.064±0.001	5.2±1.6	12

References

Alecio, M. R., Dann, M. L. & Lowe, G. (1974) *Biochem. J.* **141**, 495–501
Allen, G. & Lowe, G. (1973) *Biochem. J.* **133**, 679–686
Berger, A. & Schechter, I. (1970) *Phil. Trans. Roy. Soc. London Ser. B.* **257**, 249–264
Blumberg, S., Schechter, I. & Berger, A. (1970) *Eur. J. Biochem.* **15**, 97–102
Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1969*a*) *Data for Biochemical Research*, 2nd edn., p. 501, The Clarendon Press, Oxford
Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1969*b*) *Data for Biochemical Research*, 2nd edn., p. 529, The Clarendon Press, Oxford
Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
Glazer, A. N. & Smith, E. L. (1961) *J. Biol. Chem.* **236**, 2948–2951
Graham, C. E., Smith, E. P., Hier, S. W. & Klein, D. (1947) *J. Biol. Chem.* **168**, 711–716
Husain, S. S. & Lowe, G. (1968) *Biochem. J.* **108**, 855–859
Husain, S. S. & Lowe, G. (1969) *Biochem. J.* **114**, 279–288
Husain, S. S. & Lowe, G. (1970) *Biochem. J.* **116**, 689–692
Jori, G. & Galiazzo, G. (1971) *Photochem. Photobiol.* **14**, 607–619
Kimmel, J. R. & Smith, E. L. (1954) *J. Biol. Chem.* **207**, 515–531
Kirschenbaum, D. M. (1971) *Biochim. Biophys. Acta* **235**, 159–163
Kronman, M. J., Robbins, F. M. & Andreotti, R. E. (1967) *Biochim. Biophys. Acta* **147**, 462–472
Liu, T-Y. & Chang, Y. H. (1971) *J. Biol. Chem.* **246**, 2842–2848
Löffler, H. G. & Schneider, Fr. (1972) *Z. Naturforsch. B* **27**, 1490–1497
Lowe, G. & Yuthavong, Y. (1971) *Biochem. J.* **124**, 107–115
Matsubara, H. & Sasaki, R. M. (1969) *Biochem. Biophys. Res. Commun.* **35**, 175–181
Mitchel, R. E. J., Chaiken, I. M. & Smith, E. L. (1970) *J. Biol. Chem.* **245**, 3485–3492
Perham, R. N. (1969) *Amino Acids, Peptides and Proteins*, The Chemical Society Specialist Periodical Reports, vol. 1, pp. 31–110 (35)
Polgar, L. (1973) *Eur. J. Biochem.* **33**, 104–109
Sluyterman, L. A. Æ. & de Graaf, M. J. M. (1970) *Biochim. Biophys. Acta* 595–597
Sluyterman, L. A. Æ. & Wijdenes, J. (1970) *Biochim. Biophys. Acta* **200**, 593–595
Sluyterman, L. A. Æ. & Wijdenes, J. (1972) *Biochim. Biophys. Acta* **263**, 329–338
Steiner, R. F. (1971) *Biochemistry* **10**, 771–778
Sun Y. K. & Tsou C.-L. (1963) *Sci. Sinica* **12**, 879–884
Sun Y.-K. & Tsou C.-L. (1965) *Sci. Sinica* **14**, 749–756
Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332
Williams, A., Lucas, E. C. & Rimmer, A. R. (1972) *J. Chem. Soc. Perkin Trans. II* 621–627
Wolthers, B. G., Drenth, J., Jansonius, J. N., Koekoek, R. & Swen, H. M. (1970) *Proc. Int. Symp. Struct. Funct. Relat. Proteolytic Enzymes*, pp. 272–288, Munksgaard, Copenhagen
Yuthavong, Y. (1969) D.Phil. Thesis, University of Oxford