The Effect of Acid Proteinase Inhibitors on Chicken Pepsin

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1. The activity of chicken pepsin was partially inhibited by dimethyl-(2-hydroxy-5-nitrobenzyl)sulphonium bromide, but was unaffected by *p*-bromophenacyl bromide. 2. In the presence of Cu^{2+} , diazoacetyl norleucine methyl ester completely inactivated chicken pepsin with the incorporation of 1 mol/mol. The mechanism of the reaction was similar to that with pig pepsin. 3. Chicken pepsin was completely inactivated by 2-diazo-4-bromoacetophenone in the presence of Cu^{2+} . 4. Chicken pepsin was almost completely inactivated by 1,2-epoxy-3-(*p*-nitrophenoxy)propane at 25°C, 3–4 mol of inhibitor/mol being incorporated. The reaction at 10°C was investigated briefly. 5. Calf chymosin was inactivated by 1,2-epoxy-3-(*p*-nitrophenoxy)propane at 10°C, the incorporation of 1 mol/mol being required for complete inhibition. 6. The characteristics of the reactions of chicken pepsin with the above compounds were compared with those of other acid proteinases.

A number of acid proteinases are inactivated by two specific active-site inhibitors reacting at different loci. Diazoacetyl norleucine methyl ester inhibits pig pepsins A (EC 3.4.23.1) (Bayliss *et al.*, 1969) and C (EC 3.4.23.3) (Kay & Ryle, 1971), bovine pepsin (Meitner, 1971), calf chymosin (EC 3.4.23.4) (Chang & Takahashi, 1974) and *Penicillium janthinellum* acid proteinase (EC 3.4.23.7) (Sodek & Hofmann, 1970) by reaction with a single aspartic acid residue located in a similar sequence in each enzyme. 1,2-Epoxy-3-(*p*-nitrophenoxy)propane inhibits pig pepsin, a single aspartic acid residue different from that reacting with the diazo inhibitor being substituted (Hartsuck & Tang, 1972), and chymosin, one of the two binding sites being an aspartic acid residue in similar sequence to the reactive group in pig pepsin (Chang & Takahashi, 1974). Therefore some acid proteinases appear to have structurally similar active centres and to catalyse hydrolysis by similar mechanisms. Additional evidence for their homology is provided by the resemblance between those parts of the primary structures of pig pepsin A (Tang *et al.*, 1973), calf chymosin (Foltmann & Andersen, 1971), bovine pepsin (Harboe & Foltmann, 1973) and *P. janthinellum* acid proteinase (Harris *et al.*, 1972) and their zymogens which have been determined.

Thus mechanistically similar acid proteinases probably have similar primary structures, in turn suggesting that the tertiary structures would have resemblances, particularly in the region of the active site. Therefore, as a first approximation, a typical enzyme could serve as a structural model for the whole group. Bunn *et al.* (1970) were unable to introduce heavy atoms into chymosin crystals at defined locations. Pig pepsin has presented less difficulty and the three-dimensional structure of the molecule at about

0.5 nm resolution has been determined (Andreeva *et al.*, 1971). Chicken pepsin might be more accessible to X-ray-crystallographic analysis, as it contains a thiol group that reacts with mercurials (Bohak, 1969), suggesting a way in which a heavy atom might be fixed at specific locations in crystals. A pure chicken pepsin fraction has been obtained (Green & Llewellyn, 1973) and small crystals have been prepared (M. L. Green, unpublished work).

The purpose of the present work was to ascertain how near chicken pepsin is to being a typical acid proteinase. The mechanism of action of the enzyme was probed by using some of the pig pepsin inhibitors described. Further, the specific inhibition of calf chymosin by 1,2-epoxy-3-(*p*-nitrophenoxy)propane is described, establishing that this epoxide was a specific acid proteinase inhibitor.

Experimental

Materials

Purified chicken pepsin was prepared as described by Green & Llewellyn (1973). Crystalline calf chymosin was prepared as described by Bunn *et al.* (1971) from standard rennet powder obtained from Chr. Hansen's Laboratory Ltd., Reading, Berks., U.K., and pig pepsin (twice crystallized) was a product of Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Dimethyl-(2-hydroxy-5-nitrobenzyl)sulphonium bromide was obtained from Calbiochem, Los Angeles, Calif., U.S.A., and *p*-bromophenacyl bromide from BDH, Poole, Dorset, U.K. 2-Diazo-4-bromoacetophenone was a gift from Dr. G. C. Cheeseman. Diazoacetyl norleucine methyl ester,

synthesized as described by Kay & Ryle (1971), had a m.p. of 52°C [Kay & Ryle (1971) quote 50°C]. The product before diazotization, glycylnorleucine methyl ester hydrobromide, was submitted to elementary analysis (Found: C, 38.8; H, 6.8; N, 9.9; $C_9H_{19}N_2O_3Br$ required C, 38.2; H, 6.8; N, 9.9).

1,2-Epoxy-3-(*p*-nitrophenoxy)propane was obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A., and 1,2-dihydroxy-3-(*p*-nitrophenoxy)propane was prepared from it as described by Tang (1971). Sephadex G-25 (coarse grade) was a product of Pharmacia, Uppsala, Sweden.

Methods

Enzyme assays and protein determinations. Proteolytic activities, with haemoglobin as substrate, were determined as described previously (Green & Llewellyn, 1973). Protein concentrations were determined by the biuret micromethod of Itzhaki & Gill (1964). When a defined quantity of pepsin was required, the necessary volume of a solution of measured protein concentration was taken. The molecular weight of chicken pepsin was assumed to be 34000 (Green & Llewellyn, 1973).

Amino acid compositions of modified proteins. Protein hydrolysis and subsequent conversion of cystine derivatives into *S*-sulphocysteine were carried out as described by Liu & Inglis (1972). Amino acid analyses were performed on a Jeolco model 5AH amino acid analyser.

Reaction of pepsin with dimethyl-(2-hydroxy-5-nitrobenzyl)sulphonium bromide. Chicken pepsin (0.1 μ mol) in 1% (v/v) acetic acid (0.5 ml) was treated with reagent as described by Dopheide & Jones (1968). Modified enzyme fractions were separated as the supernatant from centrifugation (1500*g* for 15 min) of the reaction mixture (fraction 1) and as material from the residue that dissolved in 0.006 M- Na_2HPO_4 –0.044 M- NaH_2PO_4 , pH 6.0 (5 ml), in 48 h at 5°C (fraction 2). The protein from fractions 1 and 2 and a pepsin control, from which the reagent was absent, was separated from other reaction components by gel filtration on a Sephadex G-25 column (26 cm $\times$ 1.5 cm) equilibrated and eluted with 0.05 M-sodium phosphate buffer, pH 6.0. The 2-hydroxy-5-nitrobenzyl contents were determined spectrophotometrically (Barman & Koshland, 1967).

Reaction of pepsin with *p*-bromophenacyl bromide. The method was based on that of Erlanger *et al.* (1967). *p*-Bromophenacyl bromide (0.31 mg) in methanol (1 ml) was added to chicken or pig pepsin (3.1 mg) in 0.1 M-citric acid containing sufficient Na_2HPO_4 to give pH 2.65 (5 ml) and the mixture incubated at 30°C. The enzymic activity was assayed in samples of the reaction mixture withdrawn at intervals and compared with a control from which the reagent was omitted. After 24 h, the remaining

reaction mixture was applied to a Sephadex G-25 column (25.5 cm $\times$ 1.5 cm) equilibrated and eluted with 0.02 M-acetic acid. The combined protein-containing fractions, from the control and experimental samples, were adjusted to the same protein concentration and the difference spectrum was measured.

Reaction of pepsin with diazoacetylnorleucine methyl ester. Methods were based on those of Lundblad & Stein (1969). The following procedure was used unless otherwise indicated. A mixture of freshly prepared solutions of diazoacetylnorleucine methyl ester and cupric acetate in dry methanol was incubated at 25°C for 10 min. Then 4–10 vol. of pepsin (1 mg/ml) in 0.07 M-sodium acetate–0.03 M-acetic acid, pH 5.0, was added and the mixture was incubated at 25°C. The amounts of reagents were adjusted to give the maximal rate of inactivation of enzymic activity, the molar proportions of diazoacetylnorleucine methyl ester/ Cu^{2+} /pepsin varying between 90:34:1 and 186:52:1 with different preparations of the diazo compound. Diazoacetylnorleucine methyl ester was omitted from the controls. Samples for assay of enzymic activity were diluted with either 2 mM-EDTA (disodium salt) or water.

Modified protein (10 mg) was separated from the other reaction components by gel filtration on a Sephadex G-25 column (37 cm $\times$ 2 cm) equilibrated and eluted with 0.1 M-sodium acetate buffer, pH 5.0. Amino acid analysis was carried out and the number of inhibitor residues incorporated/mol of protein was calculated from the average value of the molar proportion of norleucine to the previously described (Green & Llewellyn, 1973) number of residues of aspartic acid, glutamic acid, proline, glycine, alanine, valine, leucine and phenylalanine.

Reaction of pepsin with 2-diazo-4-bromoacetophenone. Chicken pepsin was incubated with 2-diazo-4-bromoacetophenone and $CuCl_2$ as described by Erlanger *et al.* (1967), except that the temperature was 30°C. The diazo compound was omitted from the control.

Reaction of pepsin and of chymosin with 1,2-epoxy-3-(*p*-nitrophenoxy)propane. Methods were based on those of Tang (1971) and Hartsuck & Tang (1972). The following procedures were used unless otherwise indicated. Reactions were performed by incubation with shaking at 25°C for chicken pepsin and at 10°C for chymosin. The reaction mixtures, contained in conical flasks, consisted of protein (1 mg/ml), 0.33 M-sodium citrate adjusted to pH 3.5 with 0.33 M-citric acid, 0.1 mM- NaN_3 , streptomycin sulphate (50 μ g/ml), benzylpenicillin (50 μ g/ml) and solid epoxide (10 mg/ml). One drop of chloroform was also added to chicken pepsin reaction mixtures. Controls without epoxide were incubated simultaneously. At the concentrations used, the antibacterial agents had no measurable effect on the enzymic activities. Before assay or gel filtration of samples, solid epoxide was

removed by filtration through Whatman no. 1 paper on a Hirsch funnel.

Protein was separated from the other reaction components by gel filtration on a Sephadex G-25 column equilibrated and eluted with 0.1M-sodium acetate buffer, pH 5.0 (pepsin), or 5 mM-acetic acid (chymosin). For 5–10 ml and 30–45 ml samples respectively, columns of dimensions 38 cm × 2 cm and 90 cm × 2.5 cm were used. Some chicken pepsin was precipitated during the incubation and was dissolved by shaking the filtered residue with a volume of 0.012M-Na₂HPO₄–0.088M-NaH₂PO₄, pH 6.0, equal to that of the original reaction mixture. The extents of modification of the precipitated and soluble protein fractions were equal.

The extents to which the enzymes were modified were determined from the extinctions at 280 nm and 315 nm and application of equations similar to that of Tang (1971). The molar extinction coefficients used for pig pepsin and 1,2-dihydroxy-3-(*p*-nitrophenoxy)-propane were those determined by Tang (1971) and those for chicken pepsin and chymosin at 280 nm were 43 200 and 46 300 respectively. Thus the following equations were derived:

$$\text{mol of } p\text{-nitrophenoxy residues/mol of protein} \\ = (E_{315} \times a) / [E_{280} - (0.451 \times E_{315})],$$

where $a = 4.18$ for pig pepsin, 3.94 for chicken pepsin and 4.21 for chymosin.

Determination of the lability of enzymes modified with 1,2-epoxy-3-(*p*-nitrophenoxy)propane. Methods were based on those of Tang (1971). Modified enzymes were incubated at 37°C in buffers prepared by adjusting 0.1M-NH₄HCO₃ to the required pH with 0.1M-NH₃ and containing 1 mM-NaN₃. Samples (0.4–1.0 ml), removed at intervals, were applied to a Sephadex G-25 column (27 cm × 1.1 cm) equilibrated and eluted with the incubation buffer, and fractions (1 ml) were collected. Fractions containing protein were combined and the content of the *p*-nitrophenoxy moiety was determined. For identification of the other reaction product, fractions absorbing at 315 nm from the gel-filtration elutions at all incubation times were combined and evaporated to dryness on a rotary evaporator. The residue was dissolved in 5 mM-acetic acid (4 ml) and the u.v. spectrum and the R_F of the u.v.-absorbing material after t.l.c. on polyamide sheets in toluene–acetic acid (9:1, v/v) were compared with those of 1,2-dihydroxy-3-(*p*-nitrophenoxy)propane.

Results and Discussion

Reaction of chicken pepsin with dimethyl-(2-hydroxy-5-nitrobenzyl)sulphonium bromide

Two modified fractions, differing in solubility but both 46% inactivated, were obtained from chicken pepsin. Assuming that tryptophan residues had been

modified, fractions 1 and 2 contained 1.5 and 0.7 mol of 2-hydroxy-5-nitrobenzyl residues/mol respectively. However, both derivatives had higher extinctions at 408 nm than at 420 nm, suggesting that histidine rather than tryptophan might have been substituted (T. E. Barman, personal communication).

Pig pepsin was partially inhibited by the same reagent under similar conditions, two tryptophan residues being modified (Dopheide & Jones, 1968). Reports differ as to whether chymosin is partially inhibited by the similar reagent, 2-hydroxy-5-nitrobenzyl bromide (Hill & Laing, 1965; Cheeseman, 1969), but photo-oxidation of part of the tryptophan and histidine completely destroyed chymosin activity (Hill & Laing, 1965). The loss of activity of *Mucor pusillus* acid proteinase during photo-oxidation paralleled the loss of histidine, with tyrosine and tryptophan also being destroyed (Yu *et al.*, 1971). Thus either or both of tryptophan and histidine appear to be involved in the activity of those acid proteinases so far studied.

Reaction of chicken pepsin with p-bromophenacyl bromide

Treatment of chicken pepsin with *p*-bromophenacyl bromide did not affect the enzymic activity. The difference spectrum of the treated enzyme against the control had a peak at 273 nm. Assuming that the maximum extinction coefficient was the same in the protein as in the bromide derivative, 1.6 mol of *p*-bromophenacyl residues/mol were incorporated. Pig pepsin tested under similar conditions lost 69% of the enzymic activity, the difference spectrum had a peak at 268 nm and 1.3 mol of *p*-bromophenacyl residues/mol were incorporated.

Erlanger *et al.* (1967) concluded that *p*-bromophenacyl bromide partially inhibits pig pepsin by reaction with an aspartic acid residue so as to interfere sterically with the enzyme–substrate interaction. The compound partially inhibits human pepsin, but not human gastricsin (EC 3.4.23.3) (Hunkapiller *et al.*, 1970), *Rhizopus chinensis* acid proteinase (EC 3.4.23.9) (Mizobe *et al.*, 1973) nor chymosin (Cheeseman, 1969), showing that it is not a general acid proteinase inhibitor.

Reaction of chicken pepsin with diazoacetylnorleucine methyl ester

In the presence of Cu²⁺, diazoacetylnorleucine methyl ester inhibited the activity of chicken pepsin by 86 and 99% respectively after 3 and 11 min. In the absence of Cu²⁺, the enzyme was only 6% inactivated after 2 h. The addition of EDTA (disodium salt) in 10-fold molar excess over Cu²⁺ to a standard reaction mixture after incubation for 1 min maintained the activity at 46% of the original for a further 30 min.

Fig. 1. Relation between pH and the rate of inactivation of chicken pepsin by diazoacetylnorleucine methyl ester in the presence of Cu^{2+}

Pepsin was dialysed against buffers prepared from 0.1 M-sodium acetate and 0.1 M-acetic acid, the pH of the enzyme solution being measured after dialysis. The reaction time was 2 min, with the molar proportions diazoacetylnorleucine methyl ester/ Cu^{2+} /pepsin = 186:52:1. Other experimental details were standard. The results represent the average of three experiments. The points represent first-order rate constants calculated from the experimental results and the line is the calculated curve for

$$k = k_0 / \left(1 + \frac{[\text{H}^+]}{K_1} + \frac{K_2}{[\text{H}^+]} \right)$$

assuming that $k_0 = 2.8 \text{ min}^{-1}$, $\text{p}K_{a1} = 4.1$ and $\text{p}K_{a2} = 4.8$.

Therefore Cu^{2+} was essential for rapid inactivation of chicken pepsin by diazoacetylnorleucine methyl ester, as previously found for pig pepsin (Rajagopalan *et al.*, 1966).

The enzymic inactivation was more rapid if diazoacetylnorleucine methyl ester and Cu^{2+} were preincubated before the addition of pepsin than if pepsin was preincubated with Cu^{2+} before the addition of the diazo compound. This indicates that the actual inhibitor was a complex of diazoacetylnorleucine methyl ester and Cu^{2+} and that a Cu^{2+} -pepsin complex may not be an intermediate, in accord with results for pig pepsin (Lundblad & Stein, 1969).

The inactivation followed first-order kinetics at pH 4.1, 5.0 and 6.9. First-order rate constants (k) over the pH range 3.15–5.63 were calculated from the extent of inactivation of pepsin after incubation with diazoacetylnorleucine methyl ester and Cu^{2+} for 2 min at 25°C. The shape of the curve of k versus pH (Fig. 1)

Table 1. Relation between the extent of inhibition of chicken pepsin by diazoacetylnorleucine methyl ester in the presence of Cu^{2+} and the amount of norleucine incorporated into the protein

Methods are described in the Experimental section. Values for the extent of norleucine incorporation were calculated by comparison with the content of each of eight amino acids in the protein hydrolysate. The mean and S.E.M. (16 values from two experiments) are given.

Proportion of enzymic activity inhibited (%)	Norleucine incorporated (mol/mol)
0	0.00 ± 0.01
39	0.22 ± 0.03
98	1.15 ± 0.04
0	0.00 ± 0.00
57	0.62 ± 0.01
87	1.11 ± 0.03

indicated that two ionizable groups were involved in the inactivation reaction. Application of the equation

$$k = k_0 / \left(1 + \frac{[\text{H}^+]}{K_1} + \frac{K_2}{[\text{H}^+]} \right)$$

(Dixon & Webb, 1964) gave the best fit to the experimental data for $k_0 = 2.8 \text{ min}^{-1}$, $\text{p}K_{a1} = 4.1$ and $\text{p}K_{a2} = 4.8$ (Fig. 1). From these results and by analogy with pig pepsin (Lundblad & Stein, 1969), it is suggested that two carboxyl groups, one in the ionized and one in the non-ionized form, are involved in the inactivation reaction.

The stoichiometry of the reaction between diazoacetylnorleucine methyl ester and each of chicken pepsin and pepsinogen was determined. The amount of norleucine incorporated into pepsin was proportional to the extent of inactivation (Table 1), an incorporation of about 1 mol/mol being required for complete inhibition. The reaction conditions used for pepsinogen avoided activation of the zymogen and caused about 70% inactivation if pepsin was used. Pepsinogen (1 mg/ml) in 0.1 M- Na_2HPO_4 adjusted to pH 6.9 with 0.1 M- NaH_2PO_4 was incubated with the diazo compound and Cu^{2+} , the molar proportions being diazoacetylnorleucine methyl ester/ Cu^{2+} /protein = 156:54:1, for 3 h at 25°C. The treated pepsinogen was separated from the other reagents by gel filtration on a Sephadex G-25 column (33 cm × 2 cm) equilibrated and eluted with 0.1 M-sodium phosphate buffer, pH 6.9. It was activated normally at pH 2.0 and the activity of the resulting pepsin was unaffected. The mean value for the norleucine content of the pepsinogen and the S.E.M. (16 values from two experiments) were $2.10 \pm 0.06 \text{ mol/mol}$, suggesting that non-specific incorporation of inhibitor had occurred during the long incubation time. These results indicate that the inhibition of chicken pepsin was due to specific reaction at a locus in the active site

of the enzyme, in common with a number of other acid proteinases, pig pepsins A (Rajagopalan *et al.*, 1966) and C (Kay & Ryle, 1971), bovine pepsin (Meitner, 1971), human pepsin and gastricsin (Hunkapiller *et al.*, 1970), chymosin, and *R. chinensis*, *M. pusillus* and *Aspergillus saitoi* (EC 3.4.23.6) acid proteinases (Takahashi *et al.*, 1972).

The complete inhibition of chicken pepsin and pig pepsin required 20 min and less than 2 min respectively at 25°C and molar proportions of diazoacetyl-norleucine methyl ester/Cu²⁺/pepsin of 30:34:1. Differences in the rates of inactivation have been observed with other acid proteinases (Takahashi *et al.*, 1972).

Reaction of chicken pepsin with 2-diazo-4-bromoacetophenone

Chicken pepsin was 98% inhibited by incubation with 2-diazo-4-bromoacetophenone and Cu²⁺ for 24 h at 30°C. Pig pepsin (Erlanger *et al.*, 1967) and human pepsin and gastricsin (Hunkapiller *et al.*, 1970) were inhibited by the same reagent, but chymosin was not (Cheeseman, 1969). However, chymosin was inhibited by *N*-diazoacetyl-*N'*-(2,4-dinitrophenyl)ethylenediamine, which also inhibits pig pepsin (Stepanov *et al.*, 1968) and chicken pepsin (Lokshina *et al.*, 1971).

Acid proteinases vary in their rates of reaction with diazo inhibitors. Pig pepsin and chymosin appear to be extremes, with chicken pepsin intermediate. The structural requirements for compounds to be substrates appear to become more rigid in the order pig pepsin, chicken pepsin (Levchuk & Orekhovich, 1963; Bohak, 1969; Donta & Van Vunakis, 1970), chymosin (Hill, 1969). The ease of reaction of enzymes with diazo compounds may depend on the ease of access to the active site. This might be more difficult for enzymes with higher substrate specificities, since the more specific orientation at the active site might exert greater steric hindrance to inhibitor binding. In this context, it may be significant that the diazo group is situated further from the bulky side chain in the diazo compounds which inhibit chymosin than it is in 2-diazo-4-bromoacetophenone.

Reaction of chicken pepsin with 1,2-epoxy-3-(p-nitrophenoxy)propane

Pig pepsin is completely inhibited by the incorporation of 2 mol of *p*-nitrophenoxy residues/mol at 23°C and 1 mol of residue/mol at 4°C (Tang, 1971; Hartsuck & Tang, 1972). Pig pepsin was about 60% inactivated after 7 days incubation at 10°C, but chicken pepsin was only about 25% inactivated after 20 days. Both reactions followed first-order kinetics, *k* and the standard errors being $0.0051 \pm 0.0005 \text{ h}^{-1}$ (five values) for pig pepsin and $0.00048 \pm 0.00018 \text{ h}^{-1}$ (nine values) for chicken pepsin. The protein separated

from the chicken pepsin reaction mixture after 20 days incubation contained 2.0 mol of *p*-nitrophenoxy residues/mol, showing that the reaction was much less specific than it is with pig pepsin. The rate of inactivation of chicken pepsin was maximal at pH 3.5–4.5 in sodium citrate buffers at 10°C.

At 25°C, *k* and the standard errors (eight values) were $0.046 \pm 0.002 \text{ h}^{-1}$ for pig pepsin and $0.016 \pm 0.001 \text{ h}^{-1}$ for chicken pepsin. After incubation of chicken pepsin with the epoxide for 6–7 days, the mean extent of inactivation was 88 ± 1 (S.E.M., eight values)% and the content of the *p*-nitrophenoxy moiety was 3.2 ± 0.3 (S.E.M., eight values) mol of residues/mol. Thus the reaction of chicken pepsin with the epoxide was relatively slow and non-specific compared with that of pig pepsin. With human pepsin and gastricsin, calf chymosin (Tang, 1971) and *R. chinensis*, *A. saitoi* and *Trametes sanguinea* acid proteinases (Takahashi & Chang, 1973), complete inhibition at room temperature was obtained when 2 mol of *p*-nitrophenoxy residues/mol was incorporated. Comparison of the extents of inactivation and modification of chicken pepsin after various reaction times (Table 2) showed no definite difference in the rate of reaction of the epoxide with groups involved in the enzymic reaction and other groups in the enzyme.

The rate of inactivation of chicken pepsin by the epoxide followed first-order kinetics at pH values between 1.3 and 5.2, the variation of *k* with pH being shown in Fig. 2. The curve did not fit an equation based on a simple mechanism, as was also found for pig pepsin (Hartsuck & Tang, 1972). The shape of the curve was consistent with a similar mechanism of inhibition to that occurring with pig pepsin (Hartsuck

Table 2. *Relation between the extents of inactivation and modification of chicken pepsin by 1,2-epoxy-3-(p-nitrophenoxy)propane*

Pepsin was incubated with epoxide as described in the Experimental section. After the times indicated, 1.2 ml samples were withdrawn, filtered and the protein was separated from the other reaction components by gel filtration on a Sephadex G-25 column (30 cm × 1.2 cm) equilibrated and eluted with 0.1 M-sodium acetate buffer, pH 5.0. The extents of inactivation and modification of the samples were determined by comparison with controls treated similarly.

Incubation time (h)	Proportion of enzyme inactivated	<i>p</i> -Nitrophenoxy residues incorporated (mol/mol)	Extent of modification/extent of inactivation
0.0	0.00	0.00	—
21.5	0.21	1.02	4.9
45.5	0.53	1.63	3.1
69.7	0.74	2.45	3.3
143.5	0.88	3.57	4.1

Fig. 2. Relation between pH and the rate of inhibition of chicken pepsin by 1,2-epoxy-3-(*p*-nitrophenoxy)propane

The buffer in the incubation mixture was replaced by 0.1 M-HCl, 0.33 M-citric acid, or 0.33 M-citric acid adjusted to the required pH with 0.33 M-sodium citrate. The pH values are those recorded in the incubation mixture at the end of the experiment. The enzymic activity was assayed in samples of each incubation mixture after seven intervals of incubation between 0 and 146 h. The first-order rate constants and their standard errors, shown as a range on either side of each point, were calculated.

Fig. 3. Relation between pH and the rate of inhibition of chymosin by 1,2-epoxy-3-(*p*-nitrophenoxy)propane

The buffer in the incubation mixture was replaced by 0.1 M-HCl, 0.33 M-citric acid, or 0.33 M-citric acid adjusted to the required pH with 0.33 M-sodium citrate. The pH values are those recorded in the incubation mixture at the end of the experiment. The enzymic activity was assayed in samples of each incubation mixture after five intervals of incubation between 0 and 143 h. The first-order rate constants and their standard errors, shown as a range on either side of each point, were calculated.

& Tang, 1972), the reaction of the protonated form of the epoxide with one or more groups essential for enzymic activity having $\text{p}K_a$ values of less than 4.0, probably carboxylate groups.

Modified pepsin that was 94% inhibited and contained 3.0 mol of *p*-nitrophenoxy residues/mol was treated with diazoacetylnorleucine methyl ester and Cu^{2+} in the molar proportions diazo compound/ Cu^{2+} /pepsin = 168:52:1 for 4 min at 25°C. Although these conditions were sufficient to inhibit unmodified pepsin almost completely, the norleucine content of the separated protein was only 0.26 ± 0.02 (S.E.M., 16 values from two experiments) mol/mol. Thus pre-treatment with the epoxide partially inhibited the reaction of pepsin with the diazo inhibitor. This suggests that at least one of the groups involved in the reaction of the native enzyme with the diazo inhibitor had either partially reacted with the epoxide or was sterically hindered by bound residues of the epoxide. With other acid proteinases, the reactions with the two inhibitors are quite distinct. Prior incorporation of 2 mol of *p*-nitrophenoxy residues/mol did not affect the reaction of either pig pepsin (Tang, 1971) or

chymosin (Chang & Takahashi, 1973) with diazoacetylnorleucine methyl ester.

The *p*-nitrophenoxy content was not decreased by incubation of modified chicken pepsin containing 2.3 mol of *p*-nitrophenoxy residues/mol (0.82 mg/ml) at pH 8.7. On incubation of modified chicken pepsin containing 3.0 mol of *p*-nitrophenoxy residues/mol (1.7 mg/ml) in buffer- NaN_3 at pH 10.0, the content of *p*-nitrophenoxy groups fell to 1.5 mol of residues/mol in the first 22 h and maintained this value for a further 260 h. Both the non-protein reaction product and 1,2-dihydroxy-3-(*p*-nitrophenoxy)propane had peaks of maximum absorption at 315 nm, but the former had a higher extinction than the latter between 250 and 300 nm relative to the E_{315} , probably owing to the presence of impurity from the buffer. After t.l.c. both materials gave u.v.-absorbing spots of the same R_f , but the reaction product also gave a spot at the origin that fluoresced in u.v. light and was probably due to impurity from the buffer, and a minor streak of u.v.-absorbing material of slightly higher R_f than the main spot. Thus the non-protein reaction

product was primarily 1,2-dihydroxy-3-(*p*-nitrophenoxy)propane. By analogy with pig pepsin (Tang, 1971; Chen & Tang, 1972), at least some alkali-labile *p*-nitrophenoxy residues were probably released from carboxylate groups which had been esterified by reaction with the epoxide. However, alkali-stable *p*-nitrophenoxy residues probably resulted from the reaction of the epoxide with groups other than carboxylate ions. The data are consistent with the interpretation that the epoxide reacted with one carboxylate group required for enzymic activity, accompanied by non-specific and incomplete reaction at other sites, possibly including one or both of the sites involved in the reaction of the enzymes with diazoacetyl norleucine methyl ester.

Reaction of chymosin with 1,2-epoxy-3-(p-nitrophenoxy)propane

Only pig pepsin has been shown to be specifically inactivated by reaction with 1,2-epoxy-3-(*p*-nitrophenoxy)propane at a single locus in the active site. For comparative purposes, it was necessary to ascertain whether other acid proteinases are also specifically inactivated. Therefore the reaction of the epoxide with chymosin at 10°C was studied. At 25°C, chymosin is inhibited, 2 mol of *p*-nitrophenoxy residues/mol being incorporated (Chang & Takahashi, 1973).

The inactivation of chymosin by the epoxide followed first-order kinetics, k and the standard error (five values) being $0.0142 \pm 0.0005 \text{ h}^{-1}$. In four experiments, the extents of inhibition were 87, 88, 92 and 92%, with incorporations of 0.87, 0.87, 0.75 and 0.92 mol of *p*-nitrophenoxy residues/mol respectively. Therefore chymosin was specifically inhibited by reaction with 1 mol of epoxide/mol at a higher rate than pig pepsin.

At all pH values tested, the inhibition followed first-order kinetics, the variation of k with pH being shown in Fig. 3. As discussed for chicken pepsin, no equation based on a simple mechanism of inhibition was found that fitted the curve, but its course suggested that the reacting group was of pK_a 2.0 ± 0.5 .

On incubation of modified chymosin containing 0.85 mol of *p*-nitrophenoxy residues/mol (2.5 mg/ml) at pH 8.7, the content of *p*-nitrophenoxy groups fell to 0.05 mol of residues/mol after 8 days. The reaction followed first-order kinetics, k and the standard error (from six values) being $0.0129 \pm 0.0012 \text{ h}^{-1}$. From the u.v. spectrum and t.l.c., the non-protein reaction product contained only 1,2-dihydroxy-3-(*p*-nitrophenoxy)propane and material that was probably impurity from the buffer, as described for chicken pepsin. Therefore inhibition of chymosin resulted from reaction of the epoxide with an active-site carboxylate ion, probably the epoxide-reactive aspartic acid residue in the peptide isolated by Chang & Takahashi (1974). This observation adds weight to the view that 1,2-epoxy-3-(*p*-nitrophenoxy)propane is a

specific active-site inhibitor of most acid proteinases, chicken pepsin being an exception.

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