Inactivation of aspartyl proteinases by butane-2,3-dione

Modification of tryptophan and tyrosine residues and evidence against reaction of arginine residues

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Butane-2,3-dione inactivates the aspartyl proteinases from *Penicillium roqueforti* and *Penicillium caseicolum*, as well as pig pepsin, penicillopepsin and *Rhizopus* pepsin, at pH 6.0 in the presence of light but not in the dark. The inactivation is due to a photosensitized modification of tryptophan and tyrosine residues. In the dark none of the amino acid residues, not even arginine residues, is modified even after several days. In the light one arginine residue in pig pepsin is lost at a rate that is comparable with the rate of inactivation; however, the loss of the single arginine residue in the aspartyl proteinase of *P. roqueforti* and the second arginine residue of pig pepsin is slower than the loss of activity; penicillopepsin is devoid of arginine. Loss of most of the activity is accompanied by the following amino acid losses: *P. roqueforti* aspartyl proteinase, about two tryptophan and six tyrosine residues; penicillopepsin, about two tryptophan and three tyrosine residues; pig pepsin, about four tryptophan and most of the tyrosine residues. Modification of histidine residues was too slow to contribute to inactivation. None of the other residues, including half-cystine and methionine residues (when present), was modified even after prolonged incubation. The inactivation of *P. roqueforti* aspartyl proteinase and pig pepsin appears due to non-specific modification of several residues. With penicillopepsin, however, the reaction is more limited and initially affects only those tryptophan and tyrosine residues that lie in the active-site groove. In the presence of pepstatin the rate of inactivation is considerably diminished. After prolonged reaction a general structural breakdown occurs.

Some years ago Kitson & Knowles (1971) reported that phenylglyoxal at pH 5.5 decreased the activity of pig pepsin to about 45%, as measured with haemoglobin as substrate. This modification had a marked effect on the pH-dependence of the enzyme. The inactivation was accompanied by incorporation of one residue of phenylglyoxal per molecule of pepsin. The site of modification was not identified, but the authors suggested that one of the two arginine residues of pepsin had been modified, because phenylglyoxal is an arginine-specific reagent. At about the same time Huang & Tang (1972) studied the action of butane-2,3-dione, a reportedly arginine-specific reagent, on human and pig pepsin, and reported that the loss of one arginine residue was accompanied by a loss of activity. They

identified the modified arginine as that arginine residue that is located 12 residues from the C-terminus (subsequently identified as arginine-316; Tang *et al.*, 1973). Kitson & Knowles (1971) had suggested that an arginine residue might function by interacting with an active-site aspartic acid residue, thereby accounting for the low pK of one of the catalytic groups. If that were the case, then other aspartyl proteinases would be expected to contain an equivalent basic residue, since the mechanisms of the well-characterized aspartyl proteinases are very similar (Hofmann, 1974; Fruton, 1976). It was therefore interesting to find that in the amino acid sequence of penicillopepsin (Cunningham *et al.*, 1976) one of the five lysine residues (penicillopepsin is devoid of arginine) occupied a position that corresponded to one of the two arginine residues of pig pepsin, but this was arginine-308 and not the apparently important arginine-316 identified by Huang & Tang (1972). Position 308 is in a region of great sequence similarity, whereas position 316 is just outside this

Abbreviations used: Ac, acetyl; peptide T-II, Ac-Ala-Ala-Lys(Nph)-Ala-Ala-NH₂; Nph, *p*-nitrophenyl; Ac-Trp-OEt, α -*N*-acetyltryptophan ethyl ester; Ac-Tyr-NH₂, *N*-acetyltyrosine amide.

region. Modification of penicillopepsin with diethyl pyrocarbonate led to inactivation and suggested that a lysine residue has an important function in the activity (Mains, 1973; Fenje, 1979).

This suggestion received strong support from the X-ray analysis of penicillopepsin (Hsu *et al.*, 1977), which showed the unusual feature of a partly buried ion-pair between lysine-308 and aspartic acid-11 that is located at the end of the deep substrate-binding cleft near the catalytic site. An important role for this ion-pair has been suggested (James *et al.*, 1977). Position 316 on the other hand is on the outside of the molecule and a considerable distance from the active site. Since the tertiary structure of pig pepsin is very similar to that of penicillopepsin (Andreeva *et al.*, 1978), it is probable that arginine-308 rather than arginine-316 would form an ion-pair with aspartic acid-11, a residue that is conserved in all nine aspartyl proteinases investigated so far (Hofmann, 1974; Gripon *et al.*, 1977; Faerch & Foltmann, 1979). Position 308 is also highly conserved and is occupied by arginine or lysine in all the proteinases studied (Hofmann, 1974; Faerch & Foltmann, 1979). All this evidence suggests that in pepsin arginine-308 and not arginine-316 might have a role in the enzymic mechanism.

The aspartyl proteinase of *Penicillium roqueforti* is mechanistically related to pepsin and penicillopepsin (Gripon, 1976) and shows sequence homology with several aspartyl proteinases (Gripon *et al.*, 1977). It has only one arginine residue (Zevaco *et al.*, 1973), and it was therefore decided to test whether this arginine residue could be modified in a manner analogous to that of pig pepsin. When treatment with butane-2,3-dione inactivated the enzyme without concomitant loss of arginine, the possibility that the inactivation was due to the reaction of residues other than arginine became very strong. This is confirmed in the present study, which shows that butane-2,3-dione inactivates several aspartyl proteinases, but that this inactivation proceeds only in the presence of light and not in the dark, although the arginine-specific reaction proceeds readily in the dark (see Riordan, 1979). A study of the reaction of butane-2,3-dione with chymosin also led to the conclusion that inactivation is due to modification of residues other than arginine when the reaction is performed in the presence of light (A. A. Olsen & V. B. Pedersen, personal communication). However, three of the five arginine residues of chymosin react in the dark with about 20–30% reversible inactivation.

Materials and methods

Materials

Pig pepsin (3× crystallized) was from Calbiochem, San Diego, CA, U.S.A. (lot no. 300001).

Penicillopepsin was prepared as described by Hofmann (1976). *Penicillium roqueforti* aspartyl proteinase was prepared by the method of Zevaco *et al.* (1973); *Rhizopus* pepsin was from Miles Laboratories, Elkhart, IN, U.S.A.

Penicillium caseicolum aspartyl proteinase was purified as described by Lenoir *et al.* (1979). Butane-2,3-dione monomer was obtained from BDH Chemicals, Poole, Dorset, U.K. Ac-Trp-OEt and Ac-Tyr-NH₂ were from Cyclo Chemical Corp., Los Angeles, CA, U.S.A.

Methods

Amino acid analyses. Protein samples were hydrolysed with glass-distilled 5.7M-HCl *in vacuo* for 20–24 h. Additional samples were hydrolysed with 2-mercaptoethane-1-sulphonic acid for tryptophan as described by Penke *et al.* (1974). Amino acids were determined with a Beckman Spinco model 121C automated amino acid analyser. In some cases tryptophan was determined from the u.v. difference spectra, as described in the Results section.

U.v. spectra. Difference spectra were recorded with a Cary 118 spectrophotometer. Quartz cuvettes of 2 × 0.437 cm (tandem cells) were used.

C.d. spectra. C.d. spectra were recorded on a Jasco J-41A spectropolarimeter at protein concentrations of about 1.2 mg/ml (240–320 nm) or 0.15 mg/ml (200–250 nm).

Protein concentrations. Protein concentrations were determined by measuring the absorbance at 280 nm. The specific absorption coefficients (for solutions of 1 mg/ml) were: pig pepsin, 1.4; penicillopepsin, 1.35; *P. roqueforti* aspartyl proteinase, 1.31.

Enzyme activities

Aspartyl proteinases of *P. roqueforti* and *P. caseicolum* and *Rhizopus* pepsin. The activities of these enzymes were determined with haemoglobin as substrate, as follows. Haemoglobin (4%, w/v; 2 ml) in 0.1M-sodium acetate buffer, pH 3.5, was incubated for 20 min at 35°C with 2 ml of enzyme in the same buffer. The reaction was stopped by the addition of 4 ml of trichloroacetic acid (5%, w/v). After filtration, the absorbance at 280 nm was determined.

Pig pepsin. The activity of pepsin was determined in the same way, except that haemoglobin and the enzyme were dissolved in 0.1M-glycine/HCl buffer, pH 2.0.

Penicillopepsin. The activities of this enzyme were determined in three ways: (a) by the trypsinogen-activating assay as described by Hofmann (1976), (b) with bovine serum albumin as substrate (Hofmann, 1976) and (c) with peptide T-II (Hofmann *et al.*, 1979). The assay with peptide T-II was performed in 20 mM-sodium acetate buffer, pH 5.25,

at 20°C at a substrate concentration of 0.25 mM in a recording spectrophotometer at 296 nm. Initial rates were determined from the slopes.

Reaction with butane-2,3-dione. Unless otherwise noted all reactions of enzymes or model compounds with butane-2,3-dione were performed in thermostatically controlled cells either in the dark or by irradiating the cell with a G.E.C. Reflector flood tungsten lamp (150 W); the cell was placed at a distance of 34 cm from the filament of the lamp. All reactions were performed in 0.2 M-sodium phosphate buffer, pH 6.0, with protein concentrations between 0.16 and 1.1 mg/ml. Butane-2,3-dione concentrations ranged from 3 μ l/ml (34 mM) to 10 μ l/ml (113 mM).

Results

Effect of butane-2,3-dione on the activity of aspartyl proteinases

The initial experiments were performed with *P. roqueforti* aspartyl proteinase under the conditions described by Huang & Tang (1972). The enzyme was readily inactivated by butane-2,3-dione at rates comparable with those described by Huang & Tang (1972) for pepsin (Fig. 1), but the rates of inactivation varied from experiment to experiment. Subsequently, we became aware of the fact that butane-2,3-dione can undergo photosensitized reactions (Gennari & Jori, 1970; Sperling & Elad, 1971; Fujimori, 1972), and found that if the enzyme was treated with butane-2,3-dione in the dark no inactivation occurred.

vation occurred (Fig. 1, controls). Treatment of pig pepsin with butane-2,3-dione gave the same result, namely inactivation occurred only when the reaction was performed in the light (Fig. 1). Similarly, the aspartyl proteinase from *P. caseicolum* and *Rhizopus* pepsin were inactivated in the light, but not in the dark (Fig. 1). Since the well-characterized reaction of butane-2,3-dione with arginine is not photosensitized, these experiments suggested that the inactivation was due to the reaction of amino acid residues other than arginine.

This conclusion received strong support when we found that penicillopepsin, an enzyme that contains no arginine, was also inactivated by butane-2,3-dione (Fig. 2).

Effect of butane-2,3-dione on amino acid composition

Amino acid analyses of three aspartyl proteinases, *P. roqueforti* aspartyl proteinase, pig pepsin and penicillopepsin, were performed as a function of butane-2,3-dione treatment. The results are shown in Tables 1–4. In the absence of light, arginine shows only a small decrease (about 10%) in *P. roqueforti* aspartyl proteinase (Table 1) and pepsin (Table 2) over 24–48 h. No other amino acid residue is affected. However, treatment in the presence of light leads to a loss of 60–70% of arginine in both enzymes after 24 h. With *P. roqueforti* aspartyl proteinase this is clearly not related to the loss of

Fig. 1. Inactivation of aspartyl proteinases by butane-2,3-dione

The reaction mixture was incubated at 20°C in ordinary daylight: it contained enzyme (1 mg/ml) in 0.2 M-phosphate buffer, pH 6.0, and butane-2,3-dione (10 μ l/ml). Δ , Pig pepsin; $\bullet$, *P. roqueforti* aspartyl proteinase; $\blacktriangle$, *P. caseicolum* aspartyl proteinase; $\circ$, *Rhizopus* pepsin. Control solutions had the same composition but were incubated in the dark. No inactivation occurred when the enzymes were irradiated without butane-2,3-dione.

Fig. 2. Effect of butane-2,3-dione on penicillopepsin

The reaction mixture was incubated at 20°C in a quartz cuvette illuminated by two G.E.C. Reflector flood lamps (150 W) whose filaments were placed 34 cm from the centre of the cuvette. The mixture contained penicillopepsin (1 mg/ml) in 0.2 M-phosphate buffer, pH 6.0, and butane-2,3-dione (10 μ l/ml). The residual activity was determined with the following substrates: Δ and $\blacktriangle$, bovine albumin; $\circ$ and $\bullet$, trypsinogen; $\square$ and $\blacksquare$, peptide T-II. Filled-in symbols were control solutions of the same composition but incubated in the dark. No inactivation occurred when the enzyme was irradiated without butane-2,3-dione.

Table 1. *Effect of butane-2,3-dione on the amino acid composition of P. roqueforti aspartyl proteinase*

All values for the composition are given in residues/molecule. The proteinase (1 mg/ml) was incubated in 0.2 M-phosphate buffer, pH 6.0, with butane-2,3-dione (10 μ l/ml). Irradiation was from a fluorescent light-tube placed 5 cm from the reaction vial. The modified protein was hydrolysed after exhaustive dialysis. Activity was assayed with haemoglobin as substrate.

Amino acid*	Composition†	After reaction with butane-2,3-dione					
		0 min	In the light				In the dark
			90 min	180 min	360 min	1440 min	1440 min
Lysine	15	12.6	14.0	14.0	14.0	15.2	16.8
Histidine	2	1.9	2.1	1.8	2.1	1.3	1.9
Arginine	1	1.1	0.86	0.8	0.73	0.38	0.9
Methionine	0	0	0	0	0	0	0
Tyrosine	14	14.6	12.5	11.6	8.5	1.53	14.2
Leucine	21	20.3	21.9	20.5	21.2	19.9	20.7
Activity (% of that at 0 min)	—	100	29	19	10	0	100

* Only those amino acids with potentially reactive side chains are listed, except for leucine. No changes were observed in any of the other amino acids. The numbers of residues/molecule were calculated by using leucine and phenylalanine as internal standards.

† According to Zevaco *et al.* (1973).

Table 2. *Effect of butane-2,3-dione on the amino acid composition of pig pepsin*

All values are given in residues/molecule. Pepsin (1 mg/ml) was incubated in 0.2 M-phosphate buffer, pH 6.0, with butane-2,3-dione (10 μ l/ml). Irradiation was from a fluorescent light-tube placed 5 cm from the reaction vial. The modified protein was hydrolysed after exhaustive dialysis. Abbreviation: N.D., not determined.

Amino acid*	Composition†	After reaction with butane-2,3-dione						
		0 min	In the light					In the dark
			30 min	180 min	360 min	1440 min	2880 min	2880 min
Lysine	1	1.0	0.89	0.99	0.8	0.9	0.8	0.9
Histidine	1	1.0	0.94	1.12	0.87	0.7	0.5	0.95
Arginine	2	1.85	1.8	1.7	1.48	0.7	0.64	1.6
Half-cystine	6	6.4	N.D.	5.5	4.5	4.9	5.3	5.6
Methionine	4	4.1	lost	4.1	3.3	3.5	3.8	4.0
Tyrosine	16	16.2	13.3	10.4	5.5	1.7	0.7	16.1
Leucine	26	26	26.2	26	25.7	25.6	25.5	25.1
Activity (% of that at 0 min)	—	100	77	54	34	3	0	95

* See footnote * in Table 1.

† From the amino acid sequence (Tang *et al.*, 1973).

Table 3. *Effect of butane-2,3-dione on the activity and tryptophan content of pepsin and P. roqueforti aspartyl proteinase*

Conditions were as described in Table 2, except for light-intensity. Tryptophan was determined from the difference spectra of the modified versus the unmodified product. The change in absorbance at 278 nm is due primarily to tryptophan modification (see the text).

Enzyme		0 min	80 min	180 min	1320 min
Pepsin	Tryptophan modified (mol/mol)	0	2.2	3.0	4.1
	Residual activity (%)	100	72	55	15
<i>P. roqueforti</i> aspartyl proteinase	Tryptophan modified (mol/mol)	0	0.6	1.4	2.5
	Residual activity (%)	100	70	42	2

activity; thus after 360 min, when 90% of the activity is lost, 0.73 residue of arginine/molecule is still intact. With pig pepsin the results are not as clear-cut, and the loss of one arginine residue/

molecule could correlate with the loss of activity. However, in all three enzymes there is a significant loss of tyrosine and tryptophan residues.

Thus with *P. roqueforti* aspartyl proteinase the

loss of 71% activity is accompanied by a loss of 1.5 tyrosine residues/molecule, whereas a 90% activity loss involves a loss of 5.5 tyrosine residues/molecule (Table 1). The loss of tyrosine in pig pepsin is much more extensive (Table 2). About 5.6 tyrosine residues/molecule are lost at 180min, when the residual activity is still 54%. At the same time 3 tryptophan residues/molecule are lost (Table 3). With *P. roqueforti* aspartyl proteinase the loss of 1.4 tryptophan residues/molecule is accompanied by a loss of 58% activity. These results suggest that inactivation of these enzymes is due not to a specific reaction with one or two residues at or close to the active site, but rather to a random reaction of a number of residues that presumably destroys the active conformation of these enzymes in a non-specific way.

On the other hand, the loss of amino acids in penicillopepsin is much smaller. As Table 4 shows the loss of 65% activity after 180min is accompanied by a loss of less than 1 tryptophan and about 2 tyrosine residues/molecule. No other amino acids are affected. On longer incubations (360min) one more each of the tyrosine and tryptophan residues/molecule are lost and the activity falls to 12%. This indicates a considerable degree of specificity. Because of this, and also because the tertiary structure of penicillopepsin is known (Hsu *et al.*, 1977), the reaction of penicillopepsin with butane-2,3-dione was studied in some more detail.

Effect of pepstatin on reaction of butane-2,3-dione with penicillopepsin

If the loss of activity were primarily due to the reaction of butane-2,3-dione with amino acid

residues near the active site or binding site, the aspartyl proteinase inhibitor pepstatin would be expected to protect the enzyme from inactivation. The experiment in Fig. 3 shows indeed that the rate of inactivation is considerably diminished when the reaction is performed in the presence of pepstatin. [This experiment can only be performed with penicillopepsin because the trypsinogen assay allows the use of enzyme concentrations that are in the range of the inhibition constant for pepstatin (approx. 10^{-10} M), and thus the trypsinogen assays can be performed even in the presence of the inhibitor.]

Inactivation of penicillopepsin as measured with different substrates

Fig. 2 shows that when the rate of inactivation of penicillopepsin is followed by the use of three quite different substrates the inactivation curve is essentially the same. Whereas the bovine serum albumin assay measures general proteolysis, the trypsinogen assay and the assay with the hexapeptide T-II each measure the cleavage of single peptide bonds at each molecule. The parallel loss of activity therefore suggests that butane-2,3-dione causes primarily a loss of catalytic activity. This was confirmed when the K_m for partially inactivated penicillopepsin with peptide T-II as substrate was found to be the same as that of the enzyme ($K_m = 0.07 \pm 0.01$ mM at pH 5.25 at 25°C); the activity loss was accounted for by the decrease in k_{cat} .

Effect of butane-2,3-dione on c.d. spectra of penicillopepsin

We had previously shown that penicillopepsin has

Table 4. *Effect of butane-2,3-dione on the amino acid composition of penicillopepsin*

All the values for the composition are given in residues/molecule. Penicillopepsin (5 mg) was incubated in 5 ml of 0.2M-phosphate buffer, pH 6.0, at 20°C with 50 μ l of butane-2,3-dione. The reaction cuvette was placed 34 cm from the filament of a GEC Reflector flood lamp (150 W). Amino acid analyses were performed after exhaustive dialysis. The values given for the activity are each the average of the residual activity (%) obtained by the three different assay methods (Fig. 2).

Amino acid*	Composition†	0min	After reaction with butane-2,3-dione			
			In the light			In the dark
			40min	180min	360min	360min
Lysine	5	5.2	5.4	5.2	5.0	4.9
Histidine	3	3.1	3.0	3.2	2.9	3.1
Arginine	0	0	0	0	0	0
Half-cystine	2	1.9	1.8	1.75	1.85	1.85
Methionine	0	0	0	0	0	0
Tyrosine	14	14.0	13.2	11.7	11.2	13.8
Tryptophan	3	3.1	2.7	2.3	1.2	3.3
Leucine	20	20.5	21.1	19.7	19.2	20.2
Activity (% of that at 0min)	—	100	69	35	12	98

* See footnote * in Table 1.

† From the amino acid sequence (Hsu *et al.*, 1977).

Fig. 3. Effect of pepstatin on the inactivation of penicillopepsin by butane-2,3-dione

Penicillopepsin (1 mg/ml) was incubated in 0.2 M-phosphate buffer, pH 6.0, and butane-2,3-dione (10 μ l/ml) with pepstatin (○) [40 μ g/ml (2-fold molar excess)] and without pepstatin (Δ and □) at 20°C and illuminated as indicated in Fig. 2. Activities were determined with peptide T-II (□) and trypsinogen (Δ and ○) as substrates. Peptide T-II could not be used for the pepstatin-containing samples because the enzyme was completely inhibited. (The initial enzyme concentration in these assays was 75 nM.) However, the trypsinogen assay was performed at an initial enzyme concentration of 0.28 nM. At this concentration the enzyme inhibitor complex is dissociated. [Although full activities cannot be measured, the results reflect the inactivation if the inactivated enzyme still binds pepstatin. If it does not, then the apparent residual activities would be underestimated, because the ratio of inhibitor to enzyme would have increased. In this case the true loss of activity would be less than that measured. Only if the inactivated enzyme bound pepstatin more tightly (which is highly unlikely) would the residual activity be overestimated.]

several defined ellipticity bands in the 240–300 nm range (Wang *et al.*, 1974). Three of these (at 286.5, 290.5 and 293.5 nm) have been assigned to tryptophan, and the broad band at 277 nm has been ascribed to tyrosine. Fig. 4 shows the effect of butane-2,3-dione under irradiation on the c.d. spectrum between 240 and 320 nm. Curve 1 shows the spectrum of the native enzyme, curve 2 that of the enzyme after 20 min photo-oxidation, when the residual activity had decreased by only 17%. No significant changes are seen in the bands at 293.5, 290.5 and 286.5 nm, but the 277 nm band and especially the small band at 272 nm have become significantly more negative. On the other hand the ellipticity of the bands at 254, 259 and 262 nm has decreased. No change is observed at 242 nm. The

Fig. 4. Effect of butane-2,3-dione on the c.d. spectrum (240–320 nm) of penicillopepsin

Penicillopepsin (1.2 mg/ml) in 0.2 M-phosphate buffer, pH 6.0, was incubated with butane-2,3-dione (3 μ l/ml) at 25°C in c.d. spectropolarimeter cells. Irradiation was from two G.E.C. Reflector flood tungsten lamps at 34 cm from the cell. Assays were performed with peptide T-II as substrate, 1 μ l per assay being used. Curve 1 (-----), native enzyme; curve 2 (.....), after 20 min (83% activity); curve 3 (-.-.-), after 66 min (66% activity); curve 4 (—), after 122 min (38% activity).

enzyme with 66% residual activity (curve 3) shows a very marked decrease of its band at 296.5 nm, no change at 290.5 and 286.5 nm and continuing changes in the 262–280 nm region. The band at

254nm now shows a dramatic increase. All these changes are more pronounced in the enzyme with 38% residual activity (curve 4).

Although it is clearly beyond the scope of the present study to attempt a detailed interpretation of these c.d. changes, the following conclusions can be drawn. The initial effect of the reagent appears to be on tyrosine (bands 272 and 277nm), where marked changes are observed at a time when little activity is lost (curve 2). The effect on the tryptophan band at 293.5nm seems most closely correlated with the loss of activity. This is in agreement with the results of the amino acid analysis (Table 4), which shows a reasonable correlation between the loss of one tryptophan residue and activity down to about 35% residual activity. The tyrosine loss at this point is over 2 residues/molecule. This is certainly compatible with the observed c.d. changes in the range

Fig. 5. Effect of butane-2,3-dione on the c.d. spectrum (200–250 nm) of penicillopepsin

Experimental details were as given for Fig. 4. Spectra were determined in a 1 mm cell and with an enzyme diluted to 0.15 mg/ml. Curve 1 (-----), native enzyme; curve 2 (.....), after 66 min (66% activity); curve 3 (—) after 122 min (38% activity); curve 4 (- - -), after 360 min (0% activity).

254–280 nm. It is noteworthy that the bands at 242 nm (origin unknown) and at 286.5 nm (tryptophan) remain unchanged.

Fig. 5 shows the effect of butane-2,3-dione on the c.d. spectrum in the range 200–250 nm. The native enzyme (curve 1) shows a positive band at 231.5 nm and a strong negative band at 212 nm. The 231.5 nm band decreases initially (curve 2, 66% activity), but the 212 nm band is unaffected. Curve 3 (38% activity) shows a change in the 212 nm band, which suggests that the tertiary structure of the molecule is beginning to break down. Curve 4 has lost all the features of the native enzyme and is obtained from a protein with no detectable residual activity (360 min reaction).

Effect of butane-2,3-dione on u.v. spectra of aspartyl proteinases and amino acid derivatives

U.v. difference spectra of penicillopepsin treated with butane-2,3-dione are shown in Fig. 6. There is a progressive increase in absorption above 300 nm and a strong negative peak at 282 nm with a shoulder

Fig. 6. Effect of butane-2,3-dione on the u.v. difference spectrum of penicillopepsin

Penicillopepsin (1 mg/ml) in 0.2 M-phosphate buffer, pH 6.0, was incubated with butane-2,3-dione (3 μ l/ml) at 25°C in double-sector spectrophotometer cells. Irradiation was from two G.E.C. Reflector flood tungsten lamps. Irradiation was interrupted while the spectra were recorded. The curves (in order of increasing negative absorption, with maximum at 282 nm) represent incubation times of 20 min (77% activity), 45 min (64% activity), 60 min (60% activity) and 75 min (55% activity).

Fig. 7. Effect of butane-2,3-dione on the u.v. difference spectra of α -N-acetyltryptophan ethyl ester Ac-Trp-OEt (0.32mM) was incubated under the same conditions as described for penicillopepsin (Fig. 6). The curves (in order of increasing negative absorption between 265 and 295 nm) were recorded after 5, 10, 20, 30 and 40 min respectively.

Fig. 8. Effect of butane-2,3-dione on the u.v. difference spectrum of N-acetyltyrosine amide Ac-Tyr-NH₂ (0.309mM) was incubated under the same conditions as described for penicillopepsin (Fig. 6) for 15 h.

around 290nm. As expected, the spectrum is a composite of the difference spectra obtained when the model compounds Ac-Trp-OEt (Fig. 7) and Ac-Tyr-NH₂ (Fig. 8) were treated with butane-2,3-dione. The difference spectrum of the tryptophan derivative shows strong negative peaks at 293

and 280nm respectively, whereas the tyrosine derivative shows a positive peak with a maximum at 289nm. The fact that the tyrosine difference spectrum shows only a small absorbance change at 278 nm ($\Delta\epsilon_M = +260\text{M}^{-1}\cdot\text{cm}^{-1}$) whereas the tryptophan spectrum shows a change $\Delta\epsilon_M = +3190\text{M}^{-1}\cdot\text{cm}^{-1}$ was used to estimate the loss of tryptophan in pig pepsin and *P. roqueforti* aspartyl proteinase (Table 3).

The difference spectra of pig pepsin and *P. roqueforti* aspartyl proteinase were similar to that obtained for penicillopepsin, except that they show stronger positive absorbance above 300nm. This is undoubtedly due to the larger number of tyrosine residues modified in the latter enzymes compared with penicillopepsin.

Discussion

The inactivation of aspartyl proteinases by butane-2,3-dione under the conditions used by Huang & Tang (1972) is clearly due to a photosensitized reaction, and not to the specific condensation reaction with arginine. In the absence of light, arginine residues in *P. roqueforti* aspartyl proteinase and pig pepsin did not react. As a rule, reaction of arginine with butane-2,3-dione requires the presence of borate and proceeds preferably at pH 8.0 or higher (Yankeelov, 1972; Riordan, 1979). Nevertheless Huang & Tang (1972) found a loss of one arginine residue (arginine-316) that apparently correlated with the loss of activity. Since these authors did not perform the reaction in the dark, the formation of an arginine derivative was presumably due to a previously unrecognized photosensitized reaction of arginine with butane-2,3-dione. For the present study we confirmed the loss of arginine in pepsin, a loss that was accompanied by the appearance of a new peak (at 43 ml) immediately following that of arginine (at 40 ml) on the short column of the amino acid analyser (Beckman 121C). The nature of this product was not further investigated. The loss of this arginine residue, however, is not responsible for the loss of activity, since four other aspartyl proteinases, *P. roqueforti* aspartyl proteinase, penicillopepsin, *P. caseicolum* aspartyl proteinase and *Rhizopus* pepsin, also lost activity as a result of a photosensitized reaction with butane-2,3-dione. Further, the reaction with butane-2,3-dione is not specific for aspartyl proteinases. For example, Fliss & Viswanatha (1979) found that α -chymotrypsin and lysozyme also underwent rapid photo-inactivation by butane-2,3-dione. In addition, butane-2,3-dione has been used as a sensitizer in the photo-oxidation of indole derivatives (Fujimori, 1972). Unfortunately the products of this oxidation have not been identified yet. Butane-2,3-dione has

further been used for the photo-oxidation of olefines (Shimizu & Bartlett, 1976) and in a number of other studies cited by Fliss & Viswanatha (1979).

In the present study the amino acid residues that were mostly affected were tryptophan and tyrosine residues and one arginine residue in pig pepsin; losses of histidine and further arginine residues were observed only after prolonged treatment; methionine and half-cystine residues remained unaffected. The nature of the reaction products of tryptophan and tyrosine is not known. During the initial stages of the reaction tryptophan appears to be converted into a single product, as evidenced by the presence of two definite isosbestic points at 265 and 297 nm in the difference spectra shown in Fig. 7. On an extended basic column (22 cm) of the amino acid analyser initially only one product of butane-2,3-dione-treated tryptophan (elution volume 10.5 ml) is seen. (In this system tryptophan is eluted at 4.9 ml and ammonia at 100 ml.) On prolonged incubation, however, the isosbestic points of two consecutive curves shift (results not shown), and on the amino acid analyser two other new products appear that are eluted on the extended basic column at 20 ml and 35 ml. Although several studies of the photoreaction of tryptophan with butane-2,3-dione have been reported (Fliss & Viswanatha, 1979, and references cited therein), no chemical identification of the products has been made. It is unlikely, however, that the major initial product is an oxindole derivative (such as one might expect), because the u.v.-absorption spectrum (not shown) differs clearly from those of the oxindole derivatives of tryptophan described by Green & Witkop (1964).

The difference in the loss of tyrosine residues between pig pepsin and penicillopepsin is very considerable (compare Table 2 and Table 4). The reason for this is that many more tyrosine residues are exposed in pig pepsin than in penicillopepsin, although they contain a similar number (16 and 14 respectively). The X-ray structure of penicillopepsin (Hsu *et al.*, 1977) allows the calculation of the areas of the aromatic rings of the tyrosine residues that are in contact with the solvent under the static conditions of the crystal. The results obtained were as follows (M. N. G. James & A. Sielecki, personal communication). Over 30% of the surfaces of the rings of tyrosine-69, -75, -181 and -247 are in contact with solvent, the surfaces of the rings of tyrosine-175, -197, -264 and -275 are exposed to the extent of 10–20%, whereas the remaining six tyrosine residues are buried (less than 1% exposure). In contrast, it seems that most of the tyrosine residues in pepsin are at least partially exposed. Although at this time the exact location of the tyrosine residues in the tertiary structure is not known to us, a solvent perturbation study of pepsin by Nakatani *et al.* (1976) suggests that about 13

tyrosine residues are 'exposed'. The difference in tyrosine exposure between pig pepsin and penicillopepsin is also apparent from their reaction with acetylimidazole, which under identical conditions substitutes five tyrosine residues in pig pepsin and only two in penicillopepsin (P. J. Asselbergs & T. Hofmann, unpublished work).

The experiment with penicillopepsin and pepstatin (Fig. 3) indicates that the primary reaction of butane-2,3-dione takes place in the binding groove. Four potentially reactive groups are located there: tryptophan-39 and tryptophan-71, which are coplanar and are located about 0.8–1.3 nm from the catalytic groups, aspartic acid-32 and aspartic acid-215. Tyrosine-75 is also close by (0.7–1.0 nm from the catalytic site), and tyrosine-14 is situated near one end of the groove (about 0.8–1.0 nm from the catalytic aspartic acid residues.)

Tyrosine-75 is of particular interest; it is a highly conserved residue and present in all aspartyl proteinases of known sequence. It has been proposed that it functions as a proton donor during the catalytic step (James *et al.*, 1977). Tyrosine-14 may also be important; it, too, is conserved in all known sequences. Pepstatin binds tightly in this groove (K_D approx. 10^{-10} M; T. Hofmann, unpublished work) and diminishes the photo-inactivation by butane-2,3-dione, presumably by preventing access to at least some of the four groups in the binding cleft. (Pepstatin is not long enough to occupy the whole cleft.)

The c.d. studies of butane-2,3-dione-treated penicillopepsin suggest that the initial oxidation of a tyrosine and/or tryptophan leads to a conformational perturbation in the binding groove. In previous studies we had observed that when Leu-Gly-Leu, which is not a substrate but a 'competitive activator', binds to the enzyme the ellipticities of the c.d. bands at 286.5 and 290.5 nm decrease (Wang *et al.*, 1974). It is highly likely that both these bands originate from one or both tryptophan residues in the binding groove, tryptophan-39 and tryptophan-71 (Hsu *et al.*, 1977). The band at 293.5 nm also originates from the binding groove. In a recent experiment (T. Hofmann, unpublished work) we found that this band and the band at 290.5 nm are profoundly perturbed when penicillopepsin is covalently inhibited by diazo-acetylphenylalanyl-(3-phenyl)-propylamide (Irvine & Elmore, 1979). The experiment shown in Fig. 4 shows a pronounced perturbation of the bands at 293.5 and 290.5 nm. [According to Timasheff (1970) and Strickland (1974), c.d. bands >283 nm originate only from tryptophan.] The perturbation is due either to a direct modification of the residues responsible for the bands or to a secondary perturbation of non-modified residues. The complex bands between 283 and 245 nm can originate from tryptophan, tyrosine,

phenylalanine and cystine residues. The fact that the major band at 277 nm is perturbed on Leu-Gly-Leu binding suggests that it originates from a residue (most probably tyrosine) that is associated with the binding groove. It appears to be affected by butane-2,3-dione along with the band at 272 nm.

It is reasonable therefore to conclude from the c.d. experiments that the early attack of butane-2,3-dione on penicillopepsin takes place in the binding cleft. The effect is that oxidation of the two tryptophan residues and one or more tyrosine residues causes sufficient local conformational changes to decrease drastically or abolish altogether the catalytic activity. Tryptophan-71 is part of the hairpin loop that has been termed the 'tyrosine-75 flap' (James *et al.*, 1977). It is suggested that this flap is flexible and that it is implicated in conformational changes that occur on substrate binding (James, 1980). Tyrosine-75 is part of the flap and acts as the probable proton donor during the catalytic event (James *et al.*, 1977).

Although the present study shows that the experiment performed by Huang & Tang (1972) does not provide evidence for an arginine involvement in the activity of pepsin, the experiment performed by Kitson & Knowles (1971) with phenylglyoxal remains valid. These latter authors show that pepsin is partially inactivated after treatment with phenylglyoxal and that approx. 1 mol of reagent is bound/mol of enzyme. Chang (1977) reported the same result, and also found that no incorporation of phenylglyoxal occurred when pepsin was inhibited by pepstatin. This suggests that the arginine residue that is modified is part of the binding cleft and therefore is arginine-308 rather than arginine-316. The tertiary structure reported by Andreeva *et al.* (1978) shows that arginine-308 occupies a position similar to that of lysine-308 in penicillopepsin. Not surprisingly phenylglyoxal had no effect on the activity of penicillopepsin (T. Hofmann, unpublished work).

Butane-2,3-dione is an excellent and specific reagent for arginine if the reaction is carried out in the dark, at pH 8 and in the presence of borate. However, because of the lack of specificity its general use as a photo-oxidant, like that of other photosensitizing dyes that have been used for protein modification (Spikes & Livingston, 1969), is limited. Its reactions with pepsin and *P. roqueforti* aspartyl proteinase are too non-specific to yield much information. All the same, as the experiments with penicillopepsin show, its use can yield valuable information in certain favourable cases.

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