Inactivation of cysteine proteases by peptidyl epoxides: characterization of the alkylation sites on the enzyme and the inactivator

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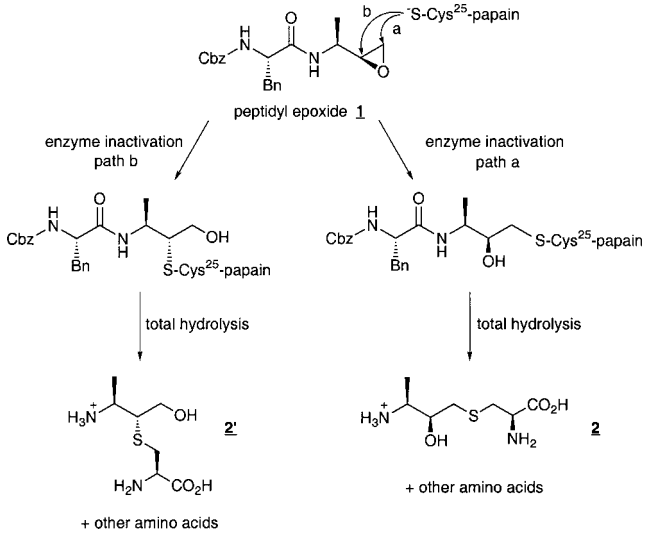
Erythro peptidyl epoxides are selective inactivators of cysteine proteases. The alkylation site, both on the enzyme papain and on the epoxide itself, was characterized. The inactivation of papain with the peptidyl epoxide *erythro* benzyloxycarbonyl-Phe-Ala-epoxide was followed by total hydrolysis by acid. Mass spectral analysis of the hydrolysate revealed, in addition to the expected amino acids, a unique signal of m/z 209 (MH^+). Its high-resolution mass spectrum and daughter peak analysis correspond to the product of alkylation on cysteine and the expected fragmentation. A similar MS pattern was obtained for a synthetic model compound corresponding to the expected hydrolysis product. A ^{13}C NMR analysis of papain inactivated by a specifically ^{13}C-labelled peptidyl epoxide indicated that the alkylation of the enzyme's cysteine residue occurs on the primary carbon of the epoxide moiety.

Key words: ^{13}C labelling, ^{13}C NMR, enzyme inhibition.

INTRODUCTION

Protease inhibitors have had an important role in the elucidation of catalytic mechanisms and structural aspects of their target enzymes [1–9]. Their potential pharmaceutical applications have also attracted much attention [10–16]. *Erythro* peptidyl epoxides (see Scheme 1) are selective cysteine protease inactivators [17,18].



Scheme 1 Inactivation of papain by peptidyl epoxides

Z-Phe-Ala-epoxide (**1**) was used to inactivate the model cystein protease papain. Incubation of the enzyme with the inactivator resulted in alkylation of the active-site cysteine residue. This reaction can proceed either via path a (alkylation at the epoxidic '*exo*' carbon) or via path b (alkylation at the epoxidic '*endo*' carbon). The inactivation complex was hydrolysed (6 M HCl, 110 °C, 24 h) and the product (either **2** or **2′** plus other amino acids) was subjected to mass spectral analysis. Abbreviations: Bn, benzyl; Cbz, benzyloxycarbonyl.

(We are aware of a single example of inactivation of a serine protease by an amino epoxide of an unusual structure [19].) They exhibit time- and concentration-dependent inhibition of cysteine proteases, but do not inhibit serine proteases. Understanding the inactivation process at the molecular level is essential for drawing mechanistic conclusions about the catalytic process of cysteine proteases and for developing novel peptidyl epoxides that can serve as inhibitors of particular cysteine proteases. Earlier studies [20] have established a 1 : 1 stoichiometry for the enzyme–inhibitor complex. The enzyme–inhibitor bond is covalent and stable under a variety of conditions, namely acidic, basic and nucleophilic and after chemical or thermal denaturation of the inhibited enzyme. Selectivity towards specific cysteine proteases was achieved by varying the amino acid sequence of the peptidyl epoxides, in correlation with good substrates or known peptide-based inhibitors. Furthermore, stereoselectivity of the inhibition process was demonstrated both with regard to the chiral centre of the epoxidic moiety and that of the P_1 amino acid (the terminology P and P′ for substrates and S and S′ for enzyme subsites is used [21]); only peptidyl epoxides derived from L-amino acids bearing an *erythro* relative configuration inhibited cysteine proteases, whereas neither the *threo* peptidyl epoxides nor a peptidyl epoxide derived from the D-amino acid at the P_1 position exhibited any inhibitory activity. These findings indicate that recognition between the enzyme and the inhibitor is similar to that between the enzyme and a corresponding substrate [17,18]. Titration experiments of the catalytic thiol suggest that the active-site cysteine residue is alkylated by the inactivator, supporting an involvement of the native catalytic machinery in the inactivation process [20]. Further analysis of the inactivation process is required. Here we characterize the alkylation site both on the cysteine protease papain and on the peptidyl epoxide inactivator. The sequence Z-Phe-Ala-epoxide (in which Z stands for benzyloxycarbonyl) was used, on the basis of sequence analyses of substrates and inhibitors [22,23] and on previous structure–activity correlation of such epoxides [17]. This peptidyl epoxide inactivates papain with second-order rate constant (k_i/K_i) of 2.1 $M^{-1} \cdot s^{-1}$.

Abbreviations used: Boc, t-butoxycarbonyl; Z, benzyloxycarbonyl.

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EXPERIMENTAL

General

Papain, amino acids, protected amino acids and protected peptides were from Sigma Chemical Company. [1-^{13}C]Alanine was from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). They were used without further purification. *N*-Boc-L-cysteine ethyl ester (in which Boc stands for t-butoxycarbonyl) was a gift from Dr. D. Mizrachi (Markowitz) (Department of Chemistry, Bar Ilan University, Ramat Gan, Israel). All synthetic compounds were characterized by ^{1}H NMR, ^{13}C NMR and MS. ^{1}H and ^{13}C NMR spectra were recorded at 300 or 600 MHz and 75 or 150 MHz respectively, in C^2HCl_3. Chemical shifts are reported in the δ scale with tetramethylsilane resonance as an internal standard. All ^{1}H NMR assignments of new compounds were supported by homonuclear two-dimensional COSY experiments; ^{13}C NMR assignments were supported either by distortionless enhancement by polarization transfer ('DEPT') or by two-dimensional hetero COSY experiments. Mass spectra were recorded in chemical ionization mode with methane as the reagent gas. TLC was performed on E. Merck 0.2 mm precoated silica-gel F-254 plates, which were viewed by either UV light or Cl_2/KI-tolidine [24]. Flash column chromatography [25] was performed on silica gel 60 (230–400 mesh ASTM; E. Merck).

Synthesis

Z-phenylalanyl-[^{13}C]alanine

1.7 g (5.5 mmol) Z-phenylalanine, 1.3 g (6.1 mmol) of dicyclohexylcarbodi-imide and 1.3 g (11.1 mmol) of *N*-hydroxysuccinimide were dissolved in 50 ml of dry tetrahydrofuran and stirred for 5 h. An aqueous solution (50 ml) of 250 mg (2.8 mmol) of [^{13}C]alanine (HCl salt) and 380 mg (2.8 mmol) of K_2CO_3 was added; the reaction was stirred for a further 20 h. Water and ether were added and the phases were separated. The aqueous phase was acidified with HCl to pH 1 and extracted twice with ethyl acetate. The organic phase was dried over $MgSO_4$, filtered and evaporated to dryness. The crude product (containing excess Z-phenylalanine) was transferred to the next reaction without further purification.

Z-phenylalanyl-alanyl-[^{13}C]epoxide (^{13}C-labelled **1**)

This was synthesized from Z-phenylalanyl-[^{13}C]alanine as described previously [17,26].

Z-phenylalanyl-alanyl (*N*-Boc-*O*-ethyl cystein-*S*-yl) methane (**4**)

To a solution of 10 mg (0.02 mmol) of Z-phenylalanyl-alanyl bromomethane (**3**) [17,26] and 5.6 mg (0.02 mmol) of *N*-Boc-L-cysteine ethyl ester in 5 ml of methanol was added 5 ml of saturated aqueous $NaHCO_3$. After 30 min of stirring at room temperature the reaction was complete, as determined by the titration of an aliquot with 5,5′-dithiobis-(2-nitrobenzoic acid) [27]. The solution was extracted with dichloromethane, dried over $MgSO_4$ and filtered. Flash chromatography with ethyl acetate/hexane (1:1, v/v) (R_F 0.5 on TLC plate) afforded 7 mg (55 % yield) of **4**.

^{1}H NMR 1.288 (t, $J = 7.2$ Hz, 3 H); 1.294 (d, $J = 7.2$ Hz, 3 H); 1.449 (s, 9 H), 2.821 (dd, $J = 13.9$, 6.5 Hz, 1H); 2.943 (dd, $J = 13.8$, 4.7 Hz, 1H); 3.043 (dd, $J = 13.8$, 7.1 Hz, 1 H); 3.122 (dd, $J = 13.8$, 6.3 Hz, 1 H); 3.316 (s, 2 H); 4.215 (q, $J = 7.1$ Hz, 2 H); 4.422 (q, $J = 6.4$ Hz, 1 H); 4.475 (q, $J = 6.7$ Hz, 1 H); 4.699 (quint, $J = 6.9$ Hz, 1 H); 5.091 (s, 2 H); 5.260 (bd, 1 H); 5.382 (d, $J = 6.6$ Hz, 1 H); 6.446 (d, $J = 6$ Hz, 1 H); 7.2–7.4 (m, 10 H). ^{13}C NMR 14.16, 17.48, 28.31, 34.44, 37.92, 38.37, 52.51, 53.04, 56.16, 61.92, 67.22, 80.28, 127.23, 128.13, 128.29, 128.58, 128.82, 129.31, 136.04, 155.20, 155.86, 170.45, 170.77, 200.88. High-resolution MS: calculated for $C_{31}H_{41}N_3O_8S$ 615.2614, found 615.2604 (M^+); MS *m/z* 516 (55); 498 (100); 390 (42); 261 (48); 200 (39); 171 (71), 118 (63).

α-Hydroxy sulphide (**5**)

A solution of **4** (7 mg, 0.01 mmol) in 2 ml of ethanol was treated with approx. 1 mg of $NaBH_4$. After 4 h, water (10 ml) was added and the solution was extracted with dichloromethane (10 ml). The organic phase was dried over $MgSO_4$, filtered and evaporated to dryness. Flash chromatography with ethyl acetate/hexane (1:1, v/v) (R_F 0.3 on TLC) afforded 5 mg (70 % yield) of **5**.

^{1}H NMR 1.25 (obscured, 3H); 1.306 (t, $J = 7.2$ Hz, 3 H); 1.457 (s, 9 H); 2.378 (dd, $J = 13.8$, 9.6 Hz, 1 H); 2.558 (dd, $J = 13.8$, 3.2 Hz, 1 H); 2.876 (dd, $J = 14.4$, 6.2 Hz, 1 H); 2.93–2.99 (m, 2 H); 3.018 (dd, $J = 14.4$, 7.8 Hz, 1 H); 3.133 (dquint., $J = 6$, 13 Hz, 1 H); 3.425 (d, $J = 8$ Hz, 1 H); 3.935 (m, 1 H); 4.237 (q, $J = 7.1$ Hz, 2 H); 4.35 (m, 1 H); 4.482 (m, 1 H); 5.094 (s, 2 H); 5.368 (m, 2 H); 5.972 (d, $J = 8.4$ Hz, 1 H); 7.1–7.4 (m, 10 H). ^{13}C NMR 14.12, 14.20, 28.33, 35.53, 37.22, 38.90, 48.68, 53.65, 56.54, 61.94, 67.13, 71.73, 127.17, 128.14, 128.28, 128.58, 128.83, 129.38, 136.15, 136.52, 155.42, 155.96, 170.85. MS (fast atom bombardment) *m/z* 618 (MH^+, 65); 553 (16); 519 (19); 518 (60); 484 (20); 461 (30); 391 (17); 369 (100); 337 (22).

Model reaction between ^{13}C-labelled peptidyl epoxide and benzyl mercaptan

Z-Phe-Ala-[^{13}C]epoxide (1 mg, 2.7 μmol, in 100 μl of CH_3CN) and 50 μl of benzyl mercaptan (53 mg, 426 μmol) were dissolved in 1 ml of water (adjusted to pH 10 with NaOH). After 48 h at room temperature, 100 μl of ^2H_2O was added and the ^{13}C NMR spectrum of the sample was recorded.

Enzymic activity

Papain activity was assayed as previously described [17].

MS analysis

Papain (EC 3.4.22.2 from Sigma) (100 mg) was activated in 10 ml of 0.05 M sodium acetate buffer, pH 5.0, containing 0.1 M KCl, 1 mM EDTA and 10 mM Na_2SO_3. After no further increase in enzymic activity was observed, the solution was loaded on an organomercury affinity chromatography column [28]. Washing with 0.05 M sodium acetate buffer, pH 5.0, containing 0.1 M KCl eluted 52 mg of inactive protein. Further elution with 100 mM potassium phosphate buffer, pH 7, containing 1 mM EDTA and 1 mM dithiothreitol afforded 30 mg of active papain; 16.8 mg of this active enzyme (2.1 mg/ml) was incubated with the inactivator Z-Phe-Ala-epoxide (compound **1**, final concentration 0.5 mM) until a complete loss of enzymic activity was observed. The solution was then dialysed against water, concentrated to approx. 1 ml and transferred to the hydrolysis tube; the water was removed by a stream of air and the dry protein was subjected to hydrolysis (6 M HCl, 110 °C, 24 h). The hydrolysate was subjected to standard MS analysis.

NMR analysis

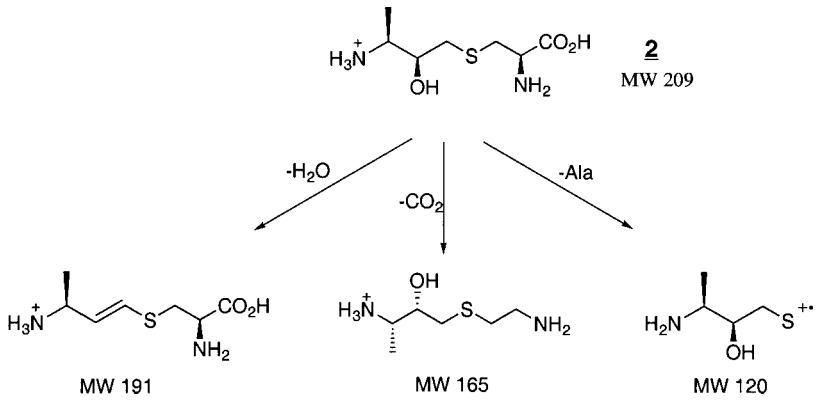
Papain (100 mg) was activated in 160 ml of 50 mM potassium phosphate buffer, pH 7.0, containing 2 mM EDTA and 1 mM dithiothreitol. After activity assay had confirmed maximal activation, the solution was concentrated (through a 10 kDa cut-off membrane) to 60 ml. A 3 ml solution of the inactivator

(10 mM in CH_3CN) was then added. After 4 h, only 2.4 % of the initial enzymic activity was detected. Further concentration to 14 ml was followed by extensive dialysis (three times against 4 litres of 1 mM HCl). The sample was further concentrated to 0.5 ml, diluted with 0.4 ml of water and 0.1 ml of ^2H_2O, and subjected to ^{13}C NMR analysis.

RESULTS

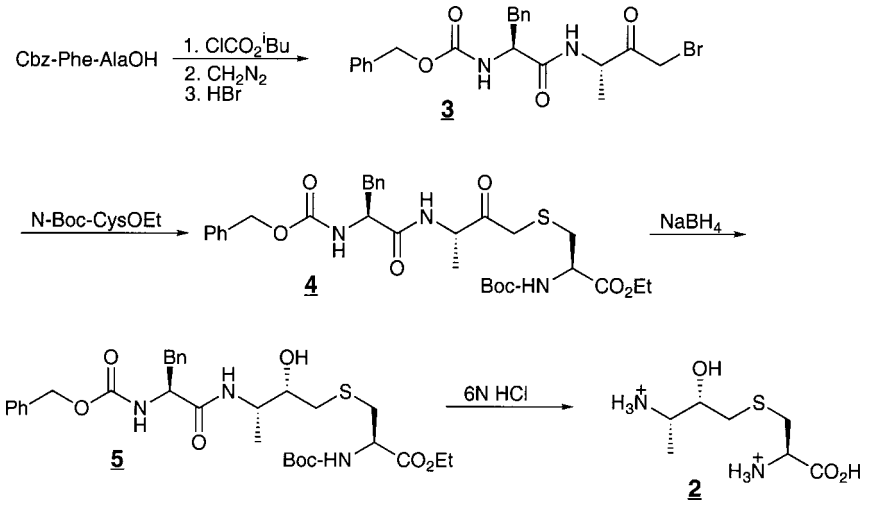
Alkylation site on the enzyme

Erythro Z-Phe-Ala-epoxide (compound **1**, Scheme 1), synthesized as described previously [17,26], was used to probe the alkylation site on the cysteine protease papain. The enzyme was activated and then purified by mercury affinity chromatography [28]. The purified active papain was inactivated by the peptidyl epoxide inhibitor. Excess unbound inhibitor was removed by dialysis, the inactivated enzyme was hydrolysed (6 M HCl, 110 °C, 24 h) and its hydrolysate was analysed by MS. Its spectrum exhibited signals corresponding to most of the natural amino acids and an additional unique signal corresponding to m/z 209 (MH^+). Its high-resolution mass spectrum fits well the molecular mass of the expected product (either **2** or **2′**) derived from the alkylation of cysteine followed by acid hydrolysis (Scheme 1). Furthermore, daughter peak analysis of the m/z 209 signal revealed three main fragments, corresponding to the elimination of water, CO_2 and alanine (Scheme 2). It should be noted that the latter signal could also correspond to fragmentation at the other C–S bond, but high-resolution MS analysis consistently supports the suggested unique fragmentation. This fragmentation pattern is consistent both with compound **2**, derived from alkylation at the '*exo*' epoxidic carbon (path a, Scheme 1), and with compound **2′**, derived from alkylation at the '*endo*' epoxidic carbon (path b, Scheme 1; the latter is not shown in Scheme 2 for reasons of clarity).



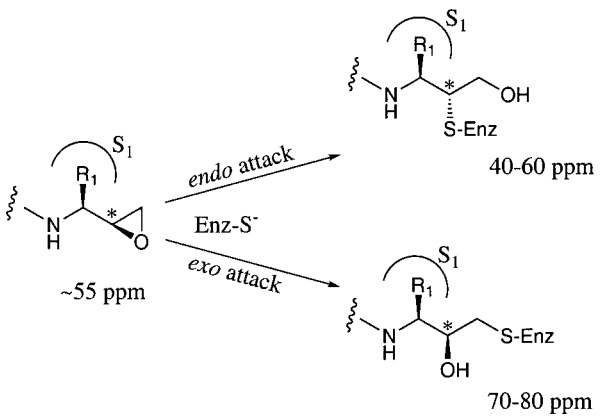
Scheme 2 MS analysis of compound 2

Papain was inactivated by the peptidyl epoxide Z-Phe-Ala-epoxide (**1**) and the inactivation complex was hydrolysed. MS analysis of the hydrolysate revealed, among other signals, a unique *m/z* 209 signal corresponding to the expected product **2** (derived from inactivation by path a in Scheme 1) or its isomer, product **2′** (derived from inactivation by path b in Scheme 1). Daughter-peak analysis identified three expected fragmentation products (with *m/z* signals of 191, 165 and 120). These fragmentation products could be derived either from **2** (as shown in the scheme) or from **2′** (not shown in the scheme, for clarity). A similar mass spectrum was also observed for an authentic sample of **2**, independently synthesized, as a control experiment.



Scheme 3 Synthesis of compound 2

An outline of the synthetic scheme towards **2**, the hydrolysis product of the expected product of alkylation of the active-site cysteine residue of papain by the peptidyl epoxide Z-Phe-Ala-epoxide. Abbreviations: Bn, benzyl; Cbz, benzyloxycarbonyl.



Scheme 4 Expected ^{13}C chemical shifts of a peptidyl epoxide and its two possible thiol alkylation products

^{13}C NMR could easily distinguish between nucleophilic attack at the methylene ('*exo*') carbon of the epoxide, yielding a primary sulphide and a secondary alcohol, or at the methine ('*endo*') epoxidic carbon, yielding a primary alcohol and a secondary sulphide. If the epoxide is ^{13}C specifically labelled at the methine carbon, the former is expected to resonate at 70–80 p.p.m., whereas the latter is expected to resonate at 40–60 p.p.m.

To confirm the MS analysis, the expected product **2** was independently synthesized and subjected to a similar MS analysis. Thus Z-Phe-AlaCH_2Br (compound **3**) was reacted with the double-protected cysteine derivative, *N*-Boc-L-cysteine ethyl ester, and the α-oxo sulphide product (compound **4**) was reduced to the corresponding α-hydroxy sulphide (compound **5**). Hydrolysis under identical conditions used for the inhibited enzyme completed the synthesis of product **2** (Scheme 3). MS analysis of this hydrolysate gave results similar to those obtained for the inactivated enzyme.

Alkylation site on the peptidyl epoxide inhibitor

^{13}C NMR had previously been employed to study covalent interactions between proteases and various inhibitors [5,29–37]. Here, the alkylation site on the epoxide itself was probed on the basis of the ^{13}C NMR analysis of the inactivation complex between papain and ^{13}C-specifically-labelled peptidyl epoxide. The *erythro* peptidyl epoxide Z-Phe-Ala-[^{13}C]epoxide, bearing a selective ^{13}C labelling on the '*endo*' carbon of the epoxide moiety, was synthesized as described previously [17,26], starting with specifically labelled [1-^{13}C]Ala. This peptidyl epoxide could alkylate the active-site cysteine residue of papain at either the epoxidic '*endo*' carbon or the epoxidic '*exo*' carbon (Schemes 1 and 4). The former results in a primary alcohol and a ^{13}C-labelled secondary sulphide, whereas the latter is characterized by a primary sulphide and a ^{13}C-labelled secondary alcohol (Scheme 4). These two possibilities should be easily distinguishable because the species involved, secondary sulphide and secondary alcohol, are expected to resonate at different frequency ranges, 40–60 p.p.m. and 70–80 p.p.m. respectively.

This distinction by the different ^{13}C NMR resonance ranges of secondary alcohols and sulphides derived from the peptidyl epoxides was verified by the analysis of appropriate model reactions. In compound **5**, the appropriate carbon, bearing the secondary hydroxy group, exhibits a chemical shift of 71.7 p.p.m. (in C^2HCl_3). A model reaction between the ^{13}C-labelled Z-Phe-Ala-epoxide and benzyl mercaptan under basic conditions afforded, in low yield, the '*exo*' attack product only. It was characterized by a strong resonance of the specifically labelled

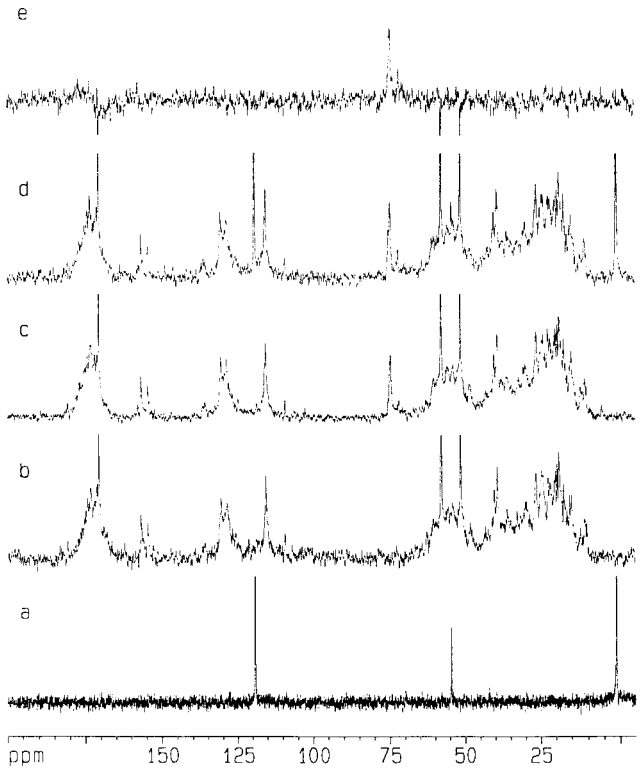


Figure 1 ^{13}C NMR spectra of inactivation complex derived from specifically labelled Z-Phe-Ala-[^{13}C]epoxide and papain at 75.4 MHz

Papain was inactivated by the specifically labelled peptidyl epoxide and, as a control, by an unlabelled identical inactivator. ^{13}C NMR spectra of the different species were recorded. Papain was dissolved in 0.9 ml of $H_2O/^2H_2O$ (9:1, v/v); free peptidyl epoxide was dissolved in 0.1 ml of CH_3CN. The resonances at 1.2 and 119.2 p.p.m. belong to CH_3CN; those at 51.6, 57.9 and 170.7 p.p.m. arise from residual EDTA. Concentrations of papain, concentrations of peptidyl epoxide and numbers of scans were respectively as follows: (**a**) 0 mM, 1.0 mM, 1000; (**b**) 0.64 mM, 0.64 mM (unlabelled peptidyl epoxide), 23000; (**c**) 0.64 mM, 0.64 mM, 16000; (**d**) 0.64 mM, 1.2 mM, 30000; (**e**) difference spectrum (**c** minus **b**). The papain concentrations mentioned were an approximation to the active enzyme (approx. 25% of the total enzyme in the sample).

carbon at 72.2 p.p.m. (in water), indicative of a secondary alcohol. No traces of an '*endo*' attack could be detected. In contrast, a secondary sulphide derived from Ep-475 (an epoxysuccinyl peptide) resonated at 53 p.p.m. [30].

The site of attack of papain's active-site cysteine residue on the Z-Phe-Ala-epoxide was then determined as follows (Figure 1). Activated papain (^{13}C NMR practically identical with that in Figure 1b) was incubated with Z-Phe-Ala-[^{13}C]epoxide. Excess free inactivator was removed from the labelled enzyme–inactivator complex by dialysis. The ^{13}C NMR spectrum of this complex revealed a new broad peak (56 Hz wide) at 74.8 p.p.m. (Figure 1c). The addition of free labelled peptidyl epoxide produced the expected 54.5 p.p.m. sharp peak (Figure 1d), demonstrating that the 74.8 p.p.m. is associated with the enzyme-bound inactivator. Finally, the ^{13}C NMR spectrum of papain inactivated by an unlabelled Z-Phe-Ala-epoxide was recorded (Figure 1b). A comparison of the spectra of the two inactivated papain samples (by labelled and unlabelled peptidyl epoxides) revealed that the only difference (besides the residual EDTA resonances) was the existence of the broad 74.8 p.p.m. peak of the labelled sample (Figure 1e).

DISCUSSION

Mechanistic conclusions about an enzyme's catalytic activity derived from selective inactivation must be supported by a detailed analysis of the inactivation process itself. In this study we characterized, at the molecular level, the inactivation of papain, the prototype cysteine protease, by peptidyl epoxides. MS was used to detect the nucleophilic enzyme residue that interacts covalently with the epoxidic moiety. ^{13}C NMR experiments identified the alkylation site on the inactivator itself.

Papain has seven cysteine residues, six of which form three disulphide bonds. Thus the catalytic cysteine residue is the only free thiol in the active enzyme [38]. Mass spectral analysis of the (hydrolysed) inactivated enzyme revealed a signal (m/z 209) that did not correspond to any of the natural protein amino acids, but fitted the expected product of cysteine alkylation (either compound **2** or **2′**; Scheme 1). Furthermore, three unique fragments of the expected compound were also observed in the mass spectrum of the inactivated enzyme hydrolysate; daughter peak analysis confirmed that they were indeed derived from the m/z 209 mother peak. This experiment therefore provided direct identification of the active-site thiol of papain as the target of alkylation by peptidyl epoxides on inactivation of the enzyme. It should be emphasized that the MS analysis (both the molecular ion and its fragmentation) is consistent with alkylation by either the '*exo*' epoxidic carbon (path a in Scheme 1) or the '*endo*' epoxidic carbon (path b in Scheme 1). It therefore clearly identifies the cysteine residue as the enzyme's alkylation site, but does not distinguish between the two possible epoxidic alkylating carbons.

Specific ^{13}C labelling of a single carbon enables its identification in a large assembly of unlabelled carbons of a protein. Thus the fate of the epoxidic '*endo*' carbon of a labelled peptidyl epoxide in the enzyme–inactivator complex was followed. Keeping in mind the above demonstration that the epoxide alkylates the active-site thiol, two possible alkylation products are possible: a secondary ^{13}C-labelled alcohol, derived from an '*exo*' attack, and a secondary ^{13}C-labelled sulphide, the product of an '*endo*' attack of the epoxide (Scheme 4). These two products are expected to differ in their ^{13}C NMR spectra. This was verified by ^{13}C NMR analysis of the products of model reactions and relevant literature data [30].

^{13}C NMR analysis of papain inactivated by peptidyl epoxide specifically labelled by ^{13}C at the epoxidic '*endo*' carbon revealed a new broad peak at 74.8 p.p.m. (Figure 1c). The peak width, arising from a short T_2 relaxation due to slow tumbling, indicates that the corresponding carbon is associated with the protein. This was further verified by the addition of excess inactivator, which exhibited the expected sharp 54.5 p.p.m. peak of the epoxidic carbon (Figure 1d). This shows that the downfield shift to 74.8 p.p.m. is indeed associated with alkylation of the enzyme and is not due to 'environmental' effects on the epoxide itself. Finally, ^{13}C NMR of the inactivation complex between papain and an unlabelled peptidyl epoxide (formed and analysed under identical conditions) did not exhibit the broad peak at 74.8 p.p.m. (Figure 1b). This unlabelled inactivation complex should be completely identical with the complex formed by the labelled inactivator, except for the specific ^{13}C-labelled single carbon. Therefore the difference spectrum of these two complexes exhibits the peak (at 74.8 p.p.m.) of the ^{13}C-labelled carbon in the inactivation complex.

This result clearly shows that the ^{13}C-labelled carbon of the enzyme–inactivator complex bears the secondary hydroxy substitution and therefore that the alkylation of the enzyme's active-site thiol occurred on the '*exo*' (primary) carbon of the inactivator's epoxide. Similar results were obtained for the inactivation of papain by Z-Gly-Leu-Phe-[^{13}C]epoxide, in which the ^{13}C-labelled carbon resonated at 69.5 p.p.m. (results not shown).

The regioselectivity of the alkylation on the epoxidic '*exo*' carbon is probably the outcome of steric hindrance at the epoxidic '*endo*' carbon. This is not surprising, considering the fact that the inhibitor bears a secondary sp^3 carbon at the position equivalent to the native substrate's planar sp^2 amide carbon. The flexibility in the active site permits the nucleophilic thiolate to attack the carbon adjacent to the would-be carbonyl carbon of a substrate. Such end products (of alkylation of the active-site thiolate by an 'adjacent' carbon) were also isolated in the inactivation of cysteine proteases by peptidyl chloromethanes [7], and probably peptidyl diazomethanes [22,23] and peptidyl fluoromethanes [22,39] (although the mechanism of alkylation is different).

The present study has identified the mode of covalent binding of peptidyl epoxides to cysteine proteases as the alkylation of the thiol of the active-site cysteine residue by the epoxidic '*exo*' carbon. Although the attack at this position slightly deviates from the normal catalytic reaction of the enzyme, the secondary alkoxide product is analogous to the catalytic tetrahedral intermediate. Thus interesting questions regarding non-covalent interactions between the oxyanion and the oxyanion hole and between the side-chain residues and the enzyme's binding site can be addressed.

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REFERENCES

1 Kaplan, A. P. and Bartlett, P. A. (1991) Biochemistry **30**, 8165–8170
2 Bartlett, P. A., Hanson, J. E. and Giannousis, P. P. (1990) J. Org. Chem. **55**, 6268–6274
3 Brady, K., Liang, T.-C. and Abeles, R. H. (1989) Biochemistry **28**, 9066–9070
4 Hanson, J. E., Kaplan, A. P. and Bartlett, P. A. (1989) Biochemistry **28**, 6294–6305
5 Liang, T.-C. and Abeles, R. H. (1987) Biochemistry **26**, 7603–7608
6 Plapp, B. V. (1982) Methods Enzymol. **87**, 469–499
7 Drenth, J., Kalk, K. H. and Swen, H. M. (1976) Biochemistry **15**, 3731–3738
8 Markland, F. S., Shaw, E. and Smith, E. L. (1968) Proc. Natl. Acad. Sci. U.S.A. **61**, 1440–1447
9 Schoellmann, G. and Shaw, E. (1963) Biochemistry **2**, 252–255
10 De Clercq, E. (1995) J. Med. Chem. **38**, 2491–2517
11 Edwards, P. D. and Berstein, P. R. (1994) Med. Res. Rev. **14**, 127–194
12 Grant, S. K., Meek, T. D., Metcalf, B. W. and Petteway, Jr., S. R. (1993) Biomed. Appl. Biotechnol. **1**, 325–353
13 Rich, D. H. (1990) in Comprehensive Medicinal Chemistry, vol. 2 (Hansch, C., Sammes, P. C. and Taylor, J. B., eds.), pp. 391–441, Pergamon Press, Oxford
14 Fischer, G. (1988) Nat. Prod. Rep. **5**, 465–495
15 Powers, J. C. and Harper, J. W. (1986) in Proteinase Inhibitors (Research Monographs in Cell and Tissue Physiology, vol. 12) (Barrett, A. J. and Salvesen, G., eds.), pp. 55–152, Elsevier, Amsterdam
16 Schnebli, H.-P. and Braun, N. J. (1986) in Proteinase Inhibitors (Research Monographs in Cell and Tissue Physiology, vol. 12) (Barrett, A. J. and Salvesen, G., eds.), pp. 613–628, Elsevier, Amsterdam
17 Albeck, A., Fluss, S. and Persky, R. (1996) J. Am. Chem. Soc. **118**, 3591–3596
18 Albeck, A., Persky, R. and Kliper, S. (1995) Bioorg. Med. Chem. Lett. **5**, 1767–1772
19 Kim, D. H., Li, Z.-H. and Lee, S. S. (1996) Bioorg. Med. Chem. Lett. **6**, 2837–2840
20 Albeck, A. and Kliper, S. (1997) Biochem. J. **322**, 879–884
21 Schechter, I. and Berger, A. (1967) Biochem. Biophys. Res. Commun. **27**, 157–162
22 Shaw, E. (1990) Adv. Enzymol. **63**, 271–347
23 Rich, D. H. (1986) in Proteinase Inhibitors (Research Monographs in Cell and Tissue Physiology, vol. 12) (Barrett, A. J. and Salvesen, G., eds.), pp. 153–178, Elsevier, Amsterdam

24 Krebs, K. G., Heusser, D. and Wimmer, H. (1969) in Thin Layer Chromatography, 2nd edn. (Stahl, E., ed.), p. 862, Springer Verlag, New York
25 Still, W. C., Kahn, M. and Mitra, A. (1978) J. Org. Chem. **43**, 2923–2925
26 Albeck, A. and Persky, R. (1994) Tetrahedron **50**, 6333–6346
27 Ellman, G. L. (1959) Arch. Biochem. Biophys. **82**, 70–77
28 Sluyterman, L. A.Æ. and Wijdenes, J. (1970) Biochim. Biophys. Acta **200**, 593–595
29 Finucane, M. D., Hudson, E. A. and Malthouse, J. P. G. (1989) Biochem. J. **258**, 853–859
30 Yabe, Y., Guillaume, D. and Rich, D. H. (1988) J. Am. Chem. Soc. **110**, 4043–4044
31 Liang, T.-C. and Abeles, R. H. (1987) Arch. Biochem. Biophys. **252**, 626–634
32 Moon, J. B., Coleman, R. S. and Hanzlik, R. P. (1986) J. Am. Chem. Soc. **108**, 1350–1351
33 Scott, A. I., Mackenzie, N. E., Malthouse, J. P. G., Primrose, W. U., Fagerness, P. E., Brisson, A., Qi, L. Z., Bode, W., Carter, C. M. and Jang, Y. J. (1986) Tetrahedron **42**, 3269–3276
34 Malthouse, J. P. G., Primrose, W. U., Mackenzie, N. E. and Scott, I. (1985) Biochemistry **24**, 3478–3487
35 Shah, D. O., Lai, K. and Gorenstein, D. G. (1984) J. Am. Chem. Soc. **106**, 4272–4273
36 Mackenzie, N. E., Malthouse, J. P. G. and Scott, A. I. (1984) Science **225**, 883–889
37 Gamcsik, M. P., Malthouse, J. P. G., Primrose, W. U., Mackenzie, N. E., Boyd, A. S. F., Russell, R. A. and Scott, I. (1983) J. Am. Chem. Soc. **105**, 6324–6325
38 Lowe, G. (1976) Tetrahedron **32**, 291–302
39 Rauber, P., Angliker, H., Walker, B. and Shaw, E. (1986) Biochem. J. **239**, 633–640

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