Mechanism of cysteine protease inactivation by peptidyl epoxides

Amnon ALBECK* and Sharon KLIPER

Department of Chemistry, Bar Ilan University, Ramat Gan 52900, Israel

Peptidyl epoxides are time- and concentration-dependent selective cysteine protease inhibitors. The lack of recovery of enzymic activity and the retention of 1 molar equivalent of radioactive inhibitor associated with the enzyme on dialysis, shown in this study, indicate that they form a covalent irreversible equimolar complex with the enzyme. It is also shown that the peptidyl epoxide inhibitors alkylate the active-site thiol. This alkylation only occurs when the enzyme is in its native conformation, as the denatured enzyme does not undergo alkylation by the inhibitor to any appreciable extent. Finally, the inactivation process is

compared with a model reaction between a peptidyl epoxide and a protected cysteine in neutral and basic aqueous media. The inactivation of cathepsin B by Cbz-Phe-(*O*-benzyl)-Thr-epoxide is accelerated by 5.5 orders of magnitude relative to the rate of the model reaction at pH 10.0 and 25 °C, and estimated to be at least 10⁸ times faster than the model reaction at pH 7.0. These results, in conjunction with the selectivity exhibited by peptidyl epoxides at all levels, point to a mechanism-based inhibition, and may have mechanistic implications regarding the catalysis carried out by cysteine proteases.

INTRODUCTION

Protease inhibitors have emerged as a class of compounds with potentially significant pharmaceutical applications [1–7]. They have also played a crucial role in mechanistic and structural studies of the various families of proteases [8–16]. Recently, we introduced peptidyl epoxides as selective cysteine protease inhibitors [17,18]. These compounds exhibit time- and concentration-dependent inhibition of cysteine proteases, but do not inhibit serine proteases. 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 with respect to both the chiral centre of the epoxidic moiety and that of the P₁ amino acid (the terminology of Schechter and Berger [19] is used, with P and P' for substrates and S and S' for enzyme subsites); 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 P₁ position exhibited any inhibitory activity.

Based on theoretical calculations, Kollman and co-workers [20,21] suggested that, whereas in serine proteases the initial catalytic reaction is a nucleophilic attack of the amide scissile bond by the serine hydroxide, in cysteine proteases protonation of the substrate precedes or occurs concomitantly with the nucleophilic attack. We proposed that the selectivity exhibited by the peptidyl epoxide inactivators supports this suggested mechanistic difference between the two families of proteases, and therefore they can be considered as mechanism-based inhibitors of cysteine proteases [17,18]. However, an understanding of the inactivation mechanism is a prerequisite for the interpretation of this inactivation selectivity in terms of different catalytic mechanisms operating in serine and cysteine proteases. Here we describe a series of studies on the mechanism of inactivation of

cysteine proteases by peptidyl epoxides the results of which support the above mechanistic hypothesis.

EXPERIMENTAL

Materials

Papain (EC 3.4.22.2), its substrate, amino acids, protected amino acids and peptides were from Sigma. NaB³H₄ (crystalline, 359.8 mCi/mmol) was from DuPont–NEN; it was mixed with 3 equiv. of unlabelled NaBH₄. The scintillation liquid used was Opti-fluor, from Packard. *N*-Boc-L-cysteine ethyl ester was prepared by Dr. Dana Markowitz, Department of Chemistry, Bar Ilan University. TLC was performed on E. Merck 0.2 mm precoated silica-gel F-254 plates.

Synthesis

Cbz-PheCH₂Br, *erythro*-Cbz-Phe-epoxide and Cbz-Gly-Leu-Phe-epoxide were synthesized by a published procedure [22]. The radiolabelled inhibitor, *erythro*-Cbz-Gly-Leu-Phe-[³H]epoxide, was synthesized similarly, using NaB³H₄ in the final reduction step. This procedure enabled radioactivity to be introduced in the last synthetic step. The radioactive peptidyl epoxide gave ¹H and ¹³C NMR spectra identical with those published in the literature for the unlabelled inhibitor. After its purification, a sample of the radioactive inhibitor was run (in two lanes and in parallel with a fully characterized unlabelled inhibitor) on a 10 cm TLC plate, and the plate was developed with either ethyl acetate/hexane (2:1, v/v; *R_F* 0.29) or chloroform/methanol (9:1, v/v; *R_F* 0.70). In both cases, half of the plate was visualized by UV light and by Cl₂/KI/toluidine [23], and the other radioactive lane was sliced into 0.5 cm strips. Each of these strips was carefully scraped into a vial, 3 ml of scintillation liquid was added, and radioactivity was measured in a liquid-scintillation β-counter. The radioactivity was shown to co-migrate with the

Abbreviations used: Boc, *t*-butoxycarbonyl; Cbz, carbobenzyloxy; DTT, dithiothreitol; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

* To whom correspondence should be addressed.

authentic unlabelled sample under these two different conditions in TLC, thus demonstrating the structure and radioactive integrity of the inhibitor.

Papain activation and assay

Protein concentration was determined by its A_{280} (ϵ 57500 $M^{-1} \cdot cm^{-1}$) [24]. The extent of enzyme activation was determined by its enzymic activity and (when required) by its free active-site thiol concentration. Papain (1–2 mg/ml) was activated by incubation at room temperature in 100 mM potassium phosphate buffer, pH 7.0, containing 2 mM cysteine or 1 mM dithiothreitol (DTT) and 2 mM EDTA, for 1–2 h. When required, the activated enzyme was loaded on a Sephadex G-15 gel-filtration column (15 cm $\times$ 1 cm), and eluted with degassed potassium phosphate buffer containing 2 mM EDTA. Fractions of volume 1 ml were collected and assayed for A_{280} . The fractions that did not contain protein were also assayed for free thiol content by using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) [25] in order to ensure separation of the protein from excess thiol-reducing agent. The fractions containing the protein were combined. Proteolytic activity (at about 55 nM enzyme) was measured by following the hydrolysis of *N*-Cbz-glycine *p*-nitrophenyl ester (25 μ M) in the same phosphate buffer at 404 nm (ϵ 9000 $M^{-1} \cdot cm^{-1}$) [26,27].

Papain inactivation

To a solution of active papain (1–2 mg/ml) was added a solution of the peptidyl epoxide in acetonitrile, such that the organic solvent volume did not exceed 10% of the total volume. Typically, final concentration of the inhibitor was 0.5 mM (for Cbz-Gly-Leu-Phe-epoxide) or 1.0 mM (for Cbz-Phe-epoxide). The solution was incubated at 25 °C for 2–3 h. A control incubation, performed under identical conditions, included all of the above constituents except for the inhibitor itself. Aliquots of 20 μ l were removed periodically and assayed for enzymic activity as described above. When necessary, the activated papain solution was passed through a Sephadex G-15 column before inactivation (as described above), and the aliquots were also assayed for free thiol concentration (by using either DTNB or 2,2'-dipyridyl disulphide [28]).

Inactivation with radioactive inhibitor

Activated papain (1–2 mg in 1 ml) was incubated with Cbz-Gly-Leu-Phe-[3H]epoxide (specific radioactivity 22.5 mCi/mmol) as described above. The inactivation solution was dialysed for 24 h in a 3 kDa cut-off dialysis tube against 3 $\times$ 2 litres of 1 mM HCl. Aliquots of 50–100 μ l were removed and diluted into 3 ml of scintillation liquid, and radioactivity was measured in a liquid-scintillation β -counter. A further 4 h dialysis and radioactivity determination were carried out to establish constant radioactivity content associated with the protein. A 10 μ l sample from the above radioactively labelled complex was diluted into 1 ml of water and dialysed for 18 h against 500 ml of 20 mM hydroxylamine, pH 8.0. A second sample was similarly dialysed against 10 mM cysteine in 100 mM potassium phosphate buffer, pH 8.0, and a third sample against water, pH 11.3. Alternatively, denatured enzyme, which was incubated with radioactive inhibitor, was separated from excess unbound inhibitor by Sephadex G-15 gel filtration, eluted with aq. 50% acetic acid. A control active enzyme sample underwent the same treatment.

Treatment with DTNB

To a solution of activated papain which was passed through a Sephadex G-15 gel-filtration column (about 0.5 mg in 0.5 ml, as

determined by its A_{280}) was added 20 μ l of an aq. 10 mM solution of DTNB. After 5 min, enzyme activity and A_{412} were determined.

Denaturation

Heat denaturation was carried out by boiling an enzyme solution for 3 min. Chemical denaturation was achieved by addition of guanidinium chloride (at 6 M final concentration). Residual enzyme activity was determined in both cases.

Model reaction

A 480 μ l buffer solution (either 100 mM potassium phosphate, pH 7.0 or 10 mM Tris/HCl, pH 10.0) was equilibrated at the desired temperature (62, 47, 38 or 25 °C) for 10 min. Then 10 μ l of 50 mM *N*-Boc-cysteine ethyl ester in methanol (20 μ l for the 38 °C incubation) was added to the buffer, followed by 10 μ l of Cbz-Phe-epoxide (50 mM in acetonitrile). Aliquots of volume 50 μ l were removed periodically, diluted with 430 μ l of phosphate buffer (pH 7.0), and assayed for free thiol concentration with DTNB. The control reaction solution was identical, except for the addition of 10 μ l of acetonitrile instead of the epoxide solution.

RESULTS

Reversibility and stoichiometry

To examine the reversibility of the inhibition of cysteine proteases by peptidyl epoxides, papain was incubated with Cbz-Gly-Leu-Phe-epoxide (loss of more than 90% of enzyme activity). The inhibited enzyme was dialysed extensively against buffer containing cysteine (1 mM) and EDTA (2 mM): no recovery of enzyme activity was detected. Active papain that was dialysed under identical conditions maintained approx. 95% of its enzyme activity.

The stoichiometry of the inactivation complex was then studied. Papain was activated and passed through a gel-filtration column to remove excess cysteine; free active-site thiol concentration was then determined. This was carried out with either DTNB or 2,2'-dipyridyl disulphide, a specific active-site titrant for the native active form of papain [28]. These two reagents consistently gave the same results, within experimental error, suggesting that the papain preparation contained only negligible

Table 1 Residual enzyme activity and radioactivity incorporated into the different forms of papain on inactivation

Papain was activated by DTT and EDTA as described in the Experimental section. It was then inactivated by the radioactive peptidyl epoxide either directly or after the appropriate treatment. Values shown are means $\pm$ S.D. for triplicate determinations. Enzyme activation may vary in different preparations. Therefore 100% enzyme activity was defined for each individual experiment. 0% enzyme activity is at background, non-enzymic hydrolysis level. Percentage radioactivity is relative to the radioactivity associated with the (untreated) inactivated enzyme of the control experiment.

Enzyme form	Enzyme activity (%)	Radioactivity (%)
Active	100	—
Inactivated	9.9 $\pm$ 4.5	93.4 $\pm$ 8.6*
Denatured after inactivation	0	94.4 $\pm$ 8.3
Treated with DTNB before inactivation	8.8 $\pm$ 3.2	9.7 $\pm$ 3.3
Denatured before inactivation	0	< 0.5

* Radioactivity associated with the protein after extensive dialysis, relative to the free thiol content of the active enzyme (before its inactivation).

Figure 1 Linear correlation between residual enzyme activity and free active-site thiol concentration on partial inactivation of papain by *erythro*-Cbz-Phe-epoxide

Papain was activated and then separated from excess free reducing thiol by gel filtration. It was then incubated with the inhibitor. At different time points along the inactivation process, aliquots were removed and assayed for both enzyme activity and titratable free thiol of the active-site cysteine.

Figure 2 Separation of incubation products of active and denatured papain from unbound radioactive peptidyl epoxide inhibitor

Separation was effected by Sephadex G-15 gel filtration. A_{280} ($\diamond$) of and radioactivity ($\circ$) associated with the native enzyme and A_{280} ($\blacklozenge$) of and radioactivity ($\bullet$) associated with the denatured enzyme are plotted against the fraction number. Protein was eluted at fractions 5–6 (native) or 6–13 (denatured), and free inhibitor was eluted at fractions 14–30. The column was 15 cm $\times$ 1 cm. Fractions of volume 1 ml were eluted with aq. 50% acetic acid at 1 ml/min.

amounts of denatured protein. It was then incubated with the radioactive inhibitor Cbz-Gly-Leu-Phe- ^{35}S epoxide. On loss of enzyme activity, it was dialysed extensively as described above. The remaining radioactivity associated with the enzyme was

determined, and correlated with the initial free active-site thiol concentration. The results showed that the molar quantity of radioactivity still associated with the enzyme was $93.4 \pm 8.6\%$ of the amount of the initial active-site thiol (Table 1). Taken together with a $9.9 \pm 4.5\%$ average residual enzyme activity remaining after the inactivation procedure, this indicates that exactly one molar equivalent of inhibitor is associated with the enzyme. This ratio did not change on further dialysis. Furthermore radioactivity was completely retained even after denaturation of the inactivated enzyme (Table 1) or after additional dialysis against acidic (HCl, pH 3.0), basic (NaOH, pH 11.3) or nucleophilic (NH_2OH or cysteine, pH 8.0) aqueous solutions.

Alkylation site

In order to identify the enzyme residue that is alkylated by the peptidyl epoxide inactivator, papain was activated, separated from excess cysteine by gel filtration, and its free active-site thiol concentration determined. It was then incubated with Cbz-Phe-epoxide. Its residual enzyme activity on partial inactivation was correlated with the remaining free active-site thiol, revealing a linear 1:1 correlation (Figure 1). When active papain was first treated with DTNB to block the active-site thiol, no incorporation of radioactivity into the enzyme was observed on its incubation with the radioactive inhibitor (Table 1).

Inhibited papain, which had no titratable free thiol, was heat denatured. This denatured (inhibited) enzyme did not exhibit any titratable free thiols either. In a control experiment, activated papain lost all its catalytic activity, but maintained most of its titratable free thiol on similar denaturation.

Active-site conformation

To examine the conformational requirements for active-site alkylation by peptidyl epoxides, activated papain was heat denatured (thus losing its catalytic activity but keeping its active-site thiol exposed to the medium) and then incubated with the radioactive inhibitor. In parallel, a sample of activated enzyme was incubated with the same radioactive inhibitor under identical conditions. When 60% inhibition of the active enzyme was measured, the two protein samples were separated from unbound radioactive inhibitor by gel filtration, and their A_{280} and radioactivity content determined. Whereas the active papain sample incorporated the expected molar equivalent of radioactivity, the denatured enzyme incorporated less than 0.5% molar equivalents of radioactivity (Table 1 and Figure 2).

Model reaction

The kinetics of cysteine protease inactivation by peptidyl epoxides were compared with that of a model reaction to estimate the

Figure 3 An expected reaction between *erythro*-Cbz-Phe-epoxide and the model thiol compound *N*-Boc-cysteine ethyl ester

Figure 4 Time course of papain inactivation and model reactions

Time course of the inactivation of papain by *erythro*-Cbz-Phe-epoxide (●) and of the interaction of the thiol model compound with the epoxide (◆) and Cbz-Phe-CH₂Br (○). Enzyme inactivation was carried out at pH 7.0, 25 °C, and the model reactions were carried out at pH 10.0, 25 °C. The enzyme concentration was 3 μM, and the concentration of all synthetic compounds (epoxide, bromide and thiol) was 1 mM.

Figure 5 Arrhenius plot for the experimental second-order rate constants of the interaction between *erythro*-Cbz-Phe-epoxide and the model thiol compound

Reactions were carried out in 10 mM Tris/HCl buffer, pH 10.0, at 62 °C, 47 °C and 38 °C (●). They were followed by periodic removal of aliquots and titration of the residual free thiol by DTNB. Extrapolation to 25 °C (○) yields the second-order rate constant at this temperature.

enzyme contribution to the alkylation process. Thus Cbz-Phe-epoxide was incubated with *N*-Boc-cysteine ethyl ester in buffer solution at both pH 7.0 and pH 10.0 (Figure 3). No loss of free thiol was detected, relative to a control incubation of the protected cysteine in similar buffer solutions over a 3 h incubation (Figure 4). In order to determine the second-order rate constant of the model reaction at pH 10.0, it was carried out (in duplicate) at 62, 47 and 38 °C. The average initial second-order rate constants of the reaction under these conditions were determined to be 0.118, 0.021 and 0.006 M⁻¹·s⁻¹ respectively (Figure 5). The second-order rate constant for the model reaction at 25 °C, extrapolated from these data, is 1.04 × 10⁻³ M⁻¹·s⁻¹. No interaction between the epoxide and the model thiol compound could be detected at pH 7.0, even at 62 °C.

Stability measurements of the epoxide were made by determining its ability to inhibit papain. At 62 °C, about 80 % of it

remained intact after 1 h incubation at both pH 7.0 and pH 10.0. At room temperature and pH 10.0, it retained approx. 95 % of its inhibitory ability after 3 h incubation.

The same model compound, *N*-Boc-cysteine ethyl ester, interacted very rapidly with Cbz-PheCH₂Br under similar conditions (1 mM concentration of the two reactants at room temperature and pH 10.0); more than 80 % of the free thiol was consumed within 2 min. A comparison of the kinetics of these two model reactions with the kinetics of papain inactivation by the same α-amino epoxide is shown in Figure 4.

DISCUSSION

This study sets the ground for an understanding of the mechanism of inactivation of cysteine proteases by peptidyl epoxides at a molecular level.

Previous studies have shown that this inhibition process is time- and concentration-dependent, and that no enzymic activity is regained on dialysis of the inhibited enzyme [17]. Here, we demonstrate the irreversible covalent nature of the inhibition process by showing that a radioactive inhibitor remains attached to the enzyme after extensive dialysis. Furthermore, radioactivity is still associated with the enzyme under acidic, basic and nucleophilic conditions and even after either thermal or chemical denaturation. These experiments also establish a 1:1 enzyme/inhibitor molar ratio in the inhibition complex, an essential feature of an affinity label or a mechanism-based enzyme inhibitor.

The good linear correlation between residual enzyme activity and titratable free active-site thiol after partial inactivation of the enzyme by the peptidyl epoxide suggests that the inhibitor-binding site at least partially overlaps the active site of the enzyme. Furthermore, pretreatment of the enzyme with DTNB (thus blocking the active-site thiol) prevents alkylation of the enzyme by the radioactive inhibitor. This result confirms that DTNB and the peptidyl epoxide inhibitor cannot simultaneously occupy the active site of the enzyme.

Papain has seven cysteine residues, six of which are blocked in three disulphide bonds, leaving only the active-site cysteine free [29]. To establish further that this free thiol is the alkylation site of peptidyl epoxide inhibitors, inhibited papain was heat denatured, and the resulting protein assayed for free thiol. If alkylation takes place on a neighbouring residue rather than on the active-site cysteine (masking the free thiol from interaction with DTNB), then denaturation of the inhibited enzyme should expose that thiol. On the other hand, if the inhibition of papain by peptidyl epoxides involves alkylation of the active-site cysteine, it should still be blocked even on denaturation. The results presented here thus strongly support alkylation of the active-site cysteine by the peptidyl epoxide inhibitors, reminiscent of alkylation of the cysteine protease active-site thiol by other known inactivators [30].

The present study not only establishes the alkylation site of papain, but also demonstrates that the enzyme has to assume an active conformation in order to interact covalently with the inhibitor. When denatured enzyme was incubated with the radioactive inhibitor for a time period that resulted in 60 % inhibition of an active enzyme under identical conditions, less than 0.5 % (molar ratio) radioactivity was incorporated. Thus it is clear that the alkylation is an enzyme-mediated process and not a simple bimolecular reaction.

The active role that the enzyme plays in the inactivation process was further demonstrated by comparing the inactivation rate with that of a model reaction between the peptidyl epoxide and a thiol compound, *N*-Boc-L-cysteine ethyl ester (Figure 3).

Figure 6 Proposed mechanism of inactivation of cysteine proteases by peptidyl epoxides

In this model, protonation precedes alkylation of the active-site thiol, but these two steps may be concerted (see the text).

This model reaction was carried out at pH 7.0, similar to the conditions of the enzyme-inactivation process, as well as at pH 10.0, at which a significant proportion of the thiol is ionized. Since the active site of the enzyme is assumed to bear the ion pair thiolate–imidazolium [31–33], these latter conditions thus more closely mimic it. In both cases, no interaction between the thiol/thiolate and the epoxide was detected even after 3 h of incubation. In order to be able to determine a rate constant for the model reaction in aqueous pH 10.0 solution, the reaction was carried out at elevated temperatures, and the rate constant at room temperature extrapolated from the appropriate temperature-dependent rates. The result obtained ($1.04 \times 10^{-3} \text{ M}^{-1} \cdot \text{s}^{-1}$) represents a 5.5 order of magnitude rate acceleration of the enzyme-inhibition process (at $330 \text{ M}^{-1} \cdot \text{s}^{-1}$) [18] over the model reaction in solution. No interaction between the epoxide and the thiol model compound was observed at pH 7.0 even at 62 °C. Thus we can estimate acceleration of the enzyme-inactivation rate relative to the rate of the bimolecular model reaction under identical conditions to be by a factor of at least 10^8 . This result can be put in perspective by comparison with the rate of interaction between the same thiol model compound and Cbz-Phe-CH₂Br, another known inhibitor of serine and cysteine proteases [3,30,34,35], in which 80 % of the free thiol was consumed within 2 min. The present results also establish that the epoxide itself is stable under the incubation conditions, especially at pH 10.0. This result is in agreement with previous observations of the stability of epoxides in neutral or basic aqueous media [36]. Thus this kinetic comparison further supports the notion that the inhibition process exhibited is not a simple bimolecular reaction, but rather a specific enzyme-mediated process.

It was previously demonstrated that papain is inhibited only by peptidyl epoxides derived from native L-amino acids at the P₁ position, and not by a compound derived from the corresponding D-amino acid. Another crucial chiral centre is that of the epoxidic methine; only the *S* (*erythro*) isomer inhibits cysteine proteases, whereas the *R* (*threo*) isomer is inactive. Amino acid sequence selectivity within the family of cysteine proteases is also exhibited in the inhibition process [18].

Thus the present results support an inactivation mechanism that is based on an enzyme-catalysed alkylation of the active-site cysteine from a Michaelis-type complex between the enzyme and the peptidyl epoxide inhibitor (Figure 6). These results and the selectivity that peptidyl epoxides exhibit by inactivating cysteine proteases but not serine proteases are mechanistically significant. We suggest that they support the proposal that in the catalytic reaction of cysteine proteases, protonation of the substrate precedes or occurs concomitantly with the nucleophilic attack on the amide scissile bond [20,21].

It is constructive to compare peptidyl epoxides with other

cysteine protease inhibitors, such as peptidyl chloromethanes, peptidyl diazomethanes and E-64 and its epoxysuccinyl analogues [30]. The second-order rate constants for inhibition of cysteine proteases by peptidyl epoxides are significantly lower than those exhibited by the corresponding peptidyl diazomethanes and peptidyl chloromethanes. This stems mainly from higher *K_i* values of the former, as previously discussed [18]. However, we demonstrated here that this rate represents a very significant acceleration of the alkylation reaction over a model chemical reaction, because of specific enzyme–inhibitor interactions.

Peptidyl epoxides are mechanistically different from the epoxysuccinyl E-64 and its synthetic analogues. Whereas the former are simple epoxides which must interact directly with the enzyme's active-site thiolate, the latter are activated epoxides. Therefore they may interact with the enzyme through a different mechanism, namely an initial attack on the carboxyl carbon, in analogy with the mechanism implicated in the inhibition of cysteine proteases by peptidyl diazomethanes [30]. Furthermore E-64 and its analogues bind in the active site of papain in a 'reversed' (retro amide) mode, relative to a substrate peptide-binding mode, thus forming a parallel β -sheet as opposed to an anti-parallel β -sheet formed between a substrate peptide and the enzyme's binding-site backbone [37,38]. It has also been suggested that some E-64 analogues may inhibit cysteine proteases by binding in the S' binding sites [39]. Thus it is not possible to draw mechanistic conclusions on the catalytic activity of these enzymes from the inhibition of cysteine proteases by E-64 and its analogues. The selectivity between serine and cysteine proteases exhibited by E-64 and peptidyl diazomethanes also does not have mechanistic implications, as it can be explained on different grounds, as previously discussed [18].

Hence we conclude that peptidyl epoxides form a distinct and unique family of selective cysteine protease inhibitors, from which meaningful conclusions on the catalytic mechanism of serine and cysteine proteases can be drawn.

In conclusion, the selective inhibition of cysteine proteases by peptidyl epoxides is time- and concentration-dependent, irreversible and covalent. It is characterized by a 1:1 enzyme/inhibitor molar ratio. The inhibition involves alkylation of the active-site cysteine, requires a native conformation of the enzyme and is significantly accelerated relative to a model reaction. Furthermore the inhibition is stereoselective with respect to two chiral centres of the inhibitor, and its amino acid sequence selectivity resembles the selectivity towards peptide substrates and other peptide-based inhibitors. These features point to an active-site-directed mechanism-based inhibition of cysteine proteases by the peptidyl epoxides, and further support mechanistic implications for their catalytic activity.

We thank Dr. D. Markowitz for the gift of *N*-Boc-cysteine ethyl ester.

REFERENCES

- 1 Rich, D. H. (1990) in *Comprehensive Medicinal Chemistry* (Hansch, C., Sammes, P. C. and Taylor, J. B., eds.), vol. 2, pp. 391–441, Pergamon Press, Oxford
- 2 Fischer, G. (1988) *Natural Product Reports*, 465–495
- 3 Powers, J. C. and Harper, J. W. (1986) in *Proteinase Inhibitors: Research Monographs in Cell and Tissue Physiology* (Barrett, A. J. and Salvesen, G., eds.), vol. 12, pp. 55–152, Elsevier, Amsterdam
- 4 Schnebli, H.-P. and Braun, N. J. (1986) in *Proteinase Inhibitors: Research Monographs in Cell and Tissue Physiology* (Barrett, A. J. and Salvesen, G., eds.), vol. 12, pp. 613–628, Elsevier, Amsterdam
- 5 Edwards, P. D. and Berstein, P. R. (1994) *Med. Res. Rev.* **14**, 127–194
- 6 De Clercq, E. (1995) *J. Med. Chem.* **38**, 2491–2517
- 7 Grant, S. K., Meek, T. D., Metcalf, B. W. and Petteway, Jr., S. R. (1993) *Biomed. Appl. Biotechnol.* **1**, 325–353
- 8 Plapp, B. V. (1982) *Methods Enzymol.* **87**, 469–499
- 9 Markland, F. S., Shaw, E. and Smith, E. L. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **61**, 1440–1447
- 10 Schoellmann, G. and Shaw, E. (1963) *Biochemistry* **2**, 252–255
- 11 Drenth, J., Kalk, K. H. and Swen, H. M. (1976) *Biochemistry* **15**, 3731–3738
- 12 Brady, K., Liang, T.-C. and Abeles, R. H. (1989) *Biochemistry* **28**, 9066–9070
- 13 Liang, T.-C. and Abeles, R. H. (1987) *Biochemistry* **26**, 7603–7608
- 14 Kaplan, A. P. and Bartlett, P. A. (1991) *Biochemistry* **30**, 8165–8170
- 15 Bartlett, P. A., Hanson, J. E. and Giannousis, P. P. (1990) *J. Org. Chem.* **55**, 6268–6274
- 16 Hanson, J. E., Kaplan, A. P. and Bartlett, P. A. (1989) *Biochemistry* **28**, 6294–6305
- 17 Albeck, A., Persky, R. and Kliper, S. (1995) *Bioorg. Med. Chem. Lett.* **5**, 1767–1772
- 18 Albeck, A., Fluss, S. and Persky, R. (1996) *J. Am. Chem. Soc.* **118**, 3591–3596
- 19 Schechter, I. and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157–162
- 20 Howard, A. E. and Kollman, P. A. (1988) *J. Am. Chem. Soc.* **110**, 7195–7200
- 21 Arad, D., Langride, R. and Kollman, P. A. (1990) *J. Am. Chem. Soc.* **112**, 491–502
- 22 Albeck, A. and Persky, R. (1994) *Tetrahedron* **50**, 6333–6346
- 23 Krebs, K. G., Heusser, D. and Wimmer, H. (1969) in *Thin Layer Chromatography*, 2nd edn. (Stahl, E., ed.), p. 862, Springer Verlag, New York
- 24 Mitchel, R. E. J., Chaiken, I. M. and Smith, E. L. (1970) *J. Biol. Chem.* **245**, 3485–3492
- 25 Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
- 26 Martin, C. J., Golubow, J. and Axelrod, A. E. (1959) *J. Biol. Chem.* **234**, 294–298
- 27 Giordano, C., Gallina, C., Consalvi, V. and Scandurra, R. (1990) *Eur. J. Med. Chem.* **25**, 479–487
- 28 Brocklehurst, K. and Little, G. (1973) *Biochem. J.* **133**, 67–80
- 29 Lowe, G. (1976) *Tetrahedron* **32**, 291–302
- 30 Rich, D. (1986) in *Proteinase Inhibitors: Research Monographs in Cell and Tissue Physiology* (Barrett, A. J. and Salvesen, G., eds.), vol. 12, pp. 153–178, Elsevier, Amsterdam
- 31 Johnson, F. A., Lewis, S. D. and Shafer, J. A. (1981) *Biochemistry* **20**, 44–48
- 32 Lewis, S. D., Johnson, F. A. and Shafer, J. A. (1981) *Biochemistry* **20**, 48–51
- 33 Polgar, L. and Halasz, P. (1982) *Biochem. J.* **207**, 1–10
- 34 Powers, J. C. (1977) *Methods Enzymol.* **46**, 197–208
- 35 Powers, J. C. (1977) in *Chemistry and Biology of Amino Acids, Peptides and Proteins* (Weinstein, B., ed.), vol. 4, pp. 66–178, Marcel Dekker, New York
- 36 Pocker, Y., Ronald, B. P. and Anderson, K. W. (1988) *J. Am. Chem. Soc.* **110**, 6492–6497
- 37 Yamamoto, D., Matsumoto, K., Ohishi, H., Ishida, T., Inoue, M., Kitamura, K. and Mizuno, H. (1991) *J. Biol. Chem.* **266**, 14771–14777
- 38 Varughese, K. I., Ahmed, F. R., Carey, P. R., Hasnain, S., Huber, C. P. and Storer, A. C. (1989) *Biochemistry* **28**, 1330–1332
- 39 Gour-Salini, B. J., Lachance, P., Plouffe, C., Storer, A. C. and Ménard, R. (1993) *J. Med. Chem.* **36**, 720–725