Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 16, pp. 1767-1772, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00312-6

DESIGN OF A NEW SELECTIVE CYSTEINE PROTEASE INACTIVATOR AND ITS MECHANISTIC IMPLICATIONS

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Abstract. Cbz-Phe-epoxide was designed as a selective inactivator of cysteine proteases. It exhibits a time- and concentration-dependent inactivation of cysteine proteases, while showing no activity towards serine proteases. The inhibition is irreversible, correlated with loss of the free active-site thiol, and its rate is at least 10^4 faster than the rate of a model reaction in solution. These results support the proposed active-site directed, protonation-dependent, mechanism-based mode of inactivation of cysteine proteases by the new inhibitor.

Proteolytic enzymes can be divided into four families, based on their active site residues and their mechanism of catalysis.[1] Two of these families, namely the serine- and cysteine proteases share many characteristics; They both utilize a covalent nucleophilic catalysis, and involve formation of an acyl enzyme and two tetrahedral intermediates along the catalytic pathway. It was recently suggested that while in serine proteases the nucleophilic attack of the serine alkoxide precedes the protonation of the leaving amino group (thus, leading to a negatively charged tetrahedral intermediate), the corresponding attack of the cysteine thiolate in cysteine proteases is subsequent to or concomitant with protonation of the substrate (leading to a neutral tetrahedral intermediate).[2] This may explain the selectivity of peptidyl diazomethanes as inhibitors of cysteine proteases.[3,4] This subtle distinction (if found to be correct) between serine- and cysteine proteases may serve as the basis for the design of selective inhibitors for cysteine proteases. Furthermore, if a compound designed to challange the proposed mechanistic difference exhibits such selectivity towards cysteine proteases - it would support the suggested modification in cysteine protease catalytic mechanism.

While epoxides are stable compounds,[5] they become highly electrophilic upon protonation. This principle was used recently in the development of an inhibitor for carboxypeptidase A, a metallo protease in which Zn^{+2} can activate the epoxidic moiety.[6] It is probably also a dominant factor in making E-64 and its derivatives efficient general inhibitors of most cysteine proteases,[7] though it was recently suggested that protonation of the epoxide in this case is carried out by a water molecule rather than by the active site histidine.[8] Weak inhibition of the cysteine protease cathepsin B was observed, upon its incubation with epoxides derived from allyl amine.[9] Other epoxides were also demonstrated to inactivate a variety of enzymes, in which activation of the epoxide is mechanistically feasible.[10] N-protected-α-amino epoxides (Figure 1), derived from the corresponding natural α-amino acids, are good analogs of the corresponding substrates of either serine or cysteine proteases and therefore are expected to bind "normally" in their respective binding sites. However, if the suggested mechanistic difference between serine- and cysteine proteases (initial nucleophilic attack in the former vs. initial protonation in the latter) is

correct, these epoxides may act as highly selective inactivators of cysteine proteases, while exhibiting very little or no inhibitory activity at all towards serine proteases. Such selective inactivation bears mechanistic significance; The fact that a given compound can form a Michaelis complex analog with both families of proteases, but inhibits only enzymes of one family, must reflect some significant mechanistic difference.

Thus, (2S,3S)-N-benzyloxycarbonyl-3-amino-1,2-epoxy-4-phenyl butane (Cbz-Phe-epoxide) was synthesized from N-Cbz-L-phenylalanine according to a short and facile procedure recently developed in our laboratory.[11] It was tested as an inhibitor of both serine and cysteine proteases (chymotrypsin and subtilisin of the former and papain and cathepsin B of the latter family). It should be emphasized that esters and amides of N-Cbz-L-phenylalanine are good substrates,[12] while the corresponding halomethyl ketones are good inactivators[13] of chymotrypsin and subtilisin. Therefore, Cbz-Phe-epoxide is expected to bind in the enzyme active site in a Michaelis-type complex. Since the dissociation constants of such compounds are rather large (in the mM range), we expect a very weak and insignificant competitive inhibition. Hence, in this study, we only examined the possibility of irreversible covalent inactivation.

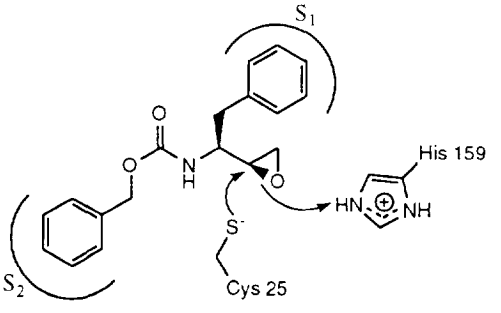

Figure 1. Schematic presentation of the proposed Michaelis-type complex between papain and the inactivator Cbz-Phe-epoxide.

Incubation of either of the serine proteases, chymotrypsin and subtilisin, with Cbz-Phe-epoxide (10 mM, 2 hours) did not yield any detectable inhibition of the enzymes. However, upon incubation with the cysteine proteases cathepsin B and papain, time- and concentration-dependent inactivation was observed (Figure 2).[14]

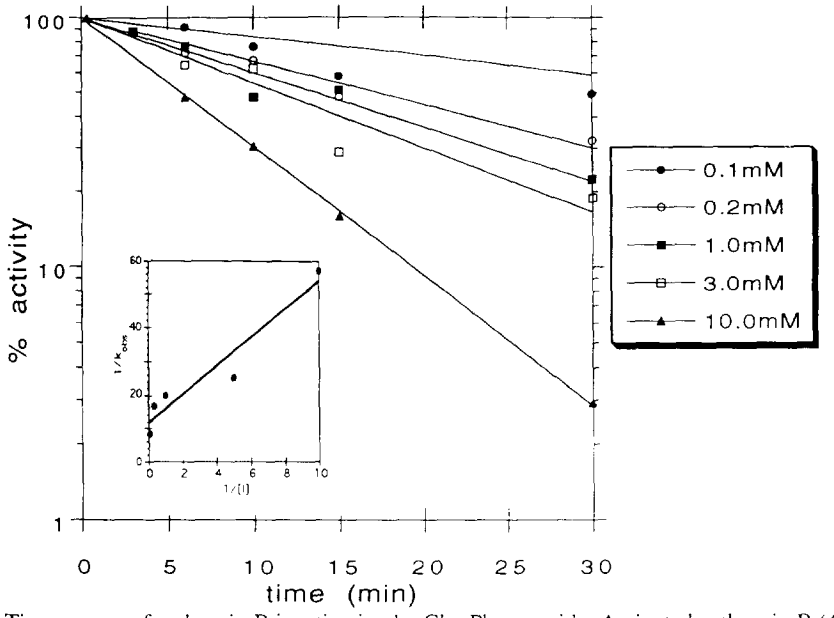

Figure 2. Time course of cathepsin B inactivation by Cbz-Phe-epoxide. Activated cathepsin B (40 mM) was incubated with the indicated concentrations of inactivator, at 25°C in 100 mM phosphate buffer pH 7.0. At time points, samples were diluted 50 fold into assay solution and residual activity measured.[14] Papain was treated similarly (data not shown).

The kinetic parameters of the inactivation process (Table 1) were derived from these data, based on the simplest kinetic scheme:

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_i} E{-}I$$

and according to equation 1:[15]

$$1/k_{obs} = (K_i/k_i)(1/[I]) + 1/k_i \qquad (1)$$

Table 1. Kinetic parameters for the inactivation of papain and cathepsin B by Cbz-Phe-epoxide.

enzyme	K_i (mM)	k_i (min^{-1})	k_i/K_i ($M^{-1}sec^{-1}$)
papain	0.574	0.208	6.03
cathepsin B	0.367	0.087	3.95

The inhibition was shown to be irreversible and covalent by both gel permeation chromatography (Sephadex G-10) and extensive dialysis [24 hours against buffer solution containing cysteine (1 mM) and EDTA (2 mM)] of inhibited papain, upon which no enzymatic activity was regained. Activated papain retained most of its enzymatic activity under identical conditions.

A good linear correlation was found between the remaining enzymatic activity and the concentration of free thiol in papain active site, as determined by DTNB (Ellman's reagent),[16] after partial inactivation of the enzyme (Figure 3). This can be interpreted in terms of shielding of the catalytic cysteine by the bound inhibitor, strongly supporting the suggested active-site directed mode of inactivation.

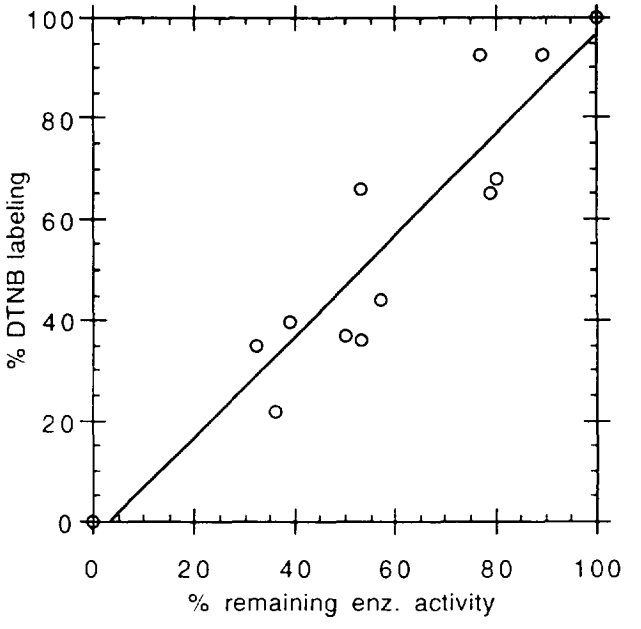

Figure 3. Correlation between residual enzymatic activity and free active-site thiol concentration, upon partial inactivation of papain by Cbz-Phe-epoxide.

The observed selectivity between serine and cysteine proteases cannot be simply explained in terms of higher nucleophilicity of thiolates, relative to alkoxides. This relative nucleophilicity holds only in protic solutions,[17] where the alkoxide is much better solvated than the thiolate, while under non solvating conditions alkoxides become better nucleophiles.[18] It is reasonable that the effective dielectric constant within the Michaelis complex is relatively low and that the nucleophile is not highly solvated, in order to facilitate the nucleophilic attack on the substrate (inhibitor). We carried out a model reaction between a thiol (N-Boc-cysteine methyl ester) and the

inhibitor Cbz-Phe-epoxide in aqueous solution[19] both at pH 7.0 and at pH 9.8[20,5] (near the thiol's pKa). Comparison of the inactivation rate with the rate of the model reaction in solution can demonstrate the active role the enzyme plays in the inactivation process. The model reaction at both pH's did not reveal any detectable interaction between the thiol and the epoxide, even after 3 hours of incubation. Similar results were obtained upon incubation of the corresponding thiolate (obtained by treatment of the model thiol with one equivalent of NaH) with the epoxide in dry methylene chloride, as a model for non-polar environment. Based on our assay sensitivity and the possible experimental inaccuracy, we can set a lower limit of 10^4 for the ratio between the second order rate constant of the enzymatic inhibition process and that of the model reaction in solution. Thus, these results represent a significant acceleration by the specific interaction of the enzyme with the inactivator relative to the rate of the corresponding model reaction in solution.

In contrast to Z-Phe-epoxide introduced here, the general cysteine protease inhibitor E-64 and its derivatives are not substrate analogs of either of the protease families. This is supported by crystallographic data which show reverse binding in the cysteine protease-inhibitor complex.[8,21] The fact that E-64 and its derivatives do not inactivate serine proteases has no direct mechanistic significance since they are not substrate analogs and their mode of binding to serine proteases (if exists) is unknown. The same applies for the dipeptide Cbz-Phe-Gly-epoxide (the best cysteine protease inhibitor in the series of epoxides derived from N-substituted allyl amines), which did not inhibit the serine protease chymotrypsin.[9] Since the latter preferentially hydrolyzes amide bonds at large hydrophobic or aromatic residues, the above inhibitors is expected to bind to chymotrypsin with its Phe side chain in the S_1 subsite, locating its epoxidic moiety in the S_1' subsite, away from the catalytic serine. In this position, the epoxide cannot act as an inactivator of chymotrypsin. Instead, we believe that it is a simple substrate, catalytically hydrolyzed to Cbz-Phe and the corresponding amino epoxide.

In summary, our results offer a new type of selective cysteine inhibitors. These results point to a specific activation of the epoxide in the cysteine protease binding site. Thus, they support the suggested mechanism in which protonation precedes the nucleophilic attack in this family of proteases,[2] justifying the term pseudo mechanism-based inactivator.[6] Further mechanistic studies of this inactivation process, as well as its extension to longer peptidyl epoxides (which will confer higher affinity and selectivity within the family of cysteine proteases, based on the specific amino acid sequence) are currently in progress in our laboratory.

Acknowledgment. This research was supported by The Israel Science Foundation administered by The Israel Academy of Sciences and Humanities.

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19. The incubation conditions were: room temperature, 100 mM phosphate buffer, and 1 mM concentration of each of the reactants. Aliquots were removed at different time points and assayed for remaining thiol concentration by DTNB. The control incubation was identical, except for the lack of the epoxide.
20. The epoxide is stable under the basic incubation conditions, as determined by its ability to inactivate papain after such incubation.
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(*Received in Belgium* 12 *April* 1995; *accepted* 13 *April* 1995)