Volume 282, number 2, 377–381 FEBS 09705 May 1991

ADONIS 001457939100414H

Functionalized *N*-aryl azetidinones as novel mechanism-based inhibitors of neutrophil elastase

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Received 21 December 1990; revised version received 18 March 1991

A functionalized *N*-aryl azetidinone has been shown to inactivate human leukocyte elastase (HLE) and porcine pancreatic elastase (PPE) by an enzyme-mediated process. The inactivation is characterized by the following kinetic constants at pH 8.0 and 37°C: $k_{inact} = 0.035\ s^{-1}$, $K_i = 1.2 \times 10^{-4}$ M for HLE, 0.08 s^{-1} and 2.7×10^{-4} M for PPE, respectively. Two parent molecules devoid of the latent leaving group failed to inactivate HLE and PPE and behaved as substrates of these enzymes. A suicide mechanism is postulated involving the formation of an acyl-enzyme and the simultaneous unmasking of a latent quinonimmonium methide ion which irreversibly reacts with an active site nucleophile. Moreover, the inhibitor is still effective at inhibiting elastase preabsorbed onto elastin.

β-Lactam; Leukocyte elastase; Suicide substrate

1. INTRODUCTION

Human leucocyte elastase (HLE), a serine protease released by the azurophilic granules of human polymorphonuclear leukocytes (PMN), degrades elastin and other connective tissue components [1]. It has been implicated in the pathogenesis of pulmonary emphysema and other inflammatory diseases such as rheumatoid arthritis and cystic fibrosis [2,3]. Synthetic inhibitors may have value in medicine for controlling tissue damage and various inflammatory or degenerative conditions mediated by elastase [4,5].

Some *β*-lactams such as *N*-tertiobutyl benzoazetidinone [6] or cephalotin [7] have been reported to be inhibitors of mammalian serine proteases such as *α*-chymotrypsin. Recently, series of neutral derivatives of cephalosporins [8,9] and monocyclic *β*-lactams [10] have been developed as mechanism-based inhibitors of HLE. Previously, we prepared a series of carboxy substituted *N*-aryl azetidinones which behaved as competitive inhibitors of bacterial serine *β*-lactamases [11]. We have now designed functionalized *N*-aryl azetidinones as mechanism-based inactivators of leukocyte elastase. One member of this new family, *N*-(2-chloromethylphenyl) 3,3-difluoro-azetidin-2-one (compound **3**, Fig. 1) was proven to be efficient at preventing the degradation of lung elastic fibers induced by elastase [12]. We present here the biochemical rationale behind the design of this new series of inhibitors of leukocyte elastase and experiments which support a mechanism-based process involving a powerful electrophilic quinonimmonium methide ion [13] (Fig. 1). In comparison, the effect on elastase activity of the unfunctionalized molecules devoid of the chloride leaving group, *N*-(2-methylphenyl) 3,3-difluoro-azetidin-2-ones (compounds **1** and **2**, Fig. 1), is analyzed. The efficiency of the inhibitor **3** towards elastase preabsorbed onto elastin is also examined.

Abbreviations: HLE, human leukocyte elastase (EC 3.4.21.37); PPE, porcine pancreatic elastase (EC 3.4.21.36); Suc-Ala_3-NA, succinyl-alanyl-alanyl-alanine *p*-nitroanilide; MeO-Suc-Ala_2-Pro-Val-NA, methoxysuccinyl-alanyl-alanyl-prolyl-valyl *p*-nitroanilide; Ac-Tyr-NA, *N*-acetyl-L-tyrosine *p*-nitroanilide; Bz-Arg-NA, benzoyl-arginyl *p*-nitroanilide; S-2251, D-valyl-L-leucyl-L-lysine *p*-nitroanilide; S-2238, D-phenylalanine-L-pipecolyl-L-arginine *p*-nitroanilide; PNA, *p*-nitrophenyl acetate; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; Z-Ala-Ala-Pro-Aala-Ala-ONp, *N*-benzyloxycarbonyl-alanyl-alanyl-prolyl-azaalanyl-*p*-nitrophenyl ester; DMSO, dimethylsulfoxide

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2. MATERIALS AND METHODS

2.1. *Materials*

PPE, HLE, bovine *α*-chymotrypsin, bovine trypsin, human plasmin and human thrombin were obtained from Serva, Elastin Products Co., Cooper Biochemicals, Sigma, Kabi Vitrum and Boehringer-Mannheim, respectively. The commercial sources of the chromogenic substrates were Sigma (Suc-Ala_3-NA for PPE), Bachem (MeO-Suc-Ala_2-Pro-Val-NA for HLE), Serva (Ac-Tyr-NA for chymotrypsin), Boehringer-Mannheim (Bz-Arg-NA for trypsin),

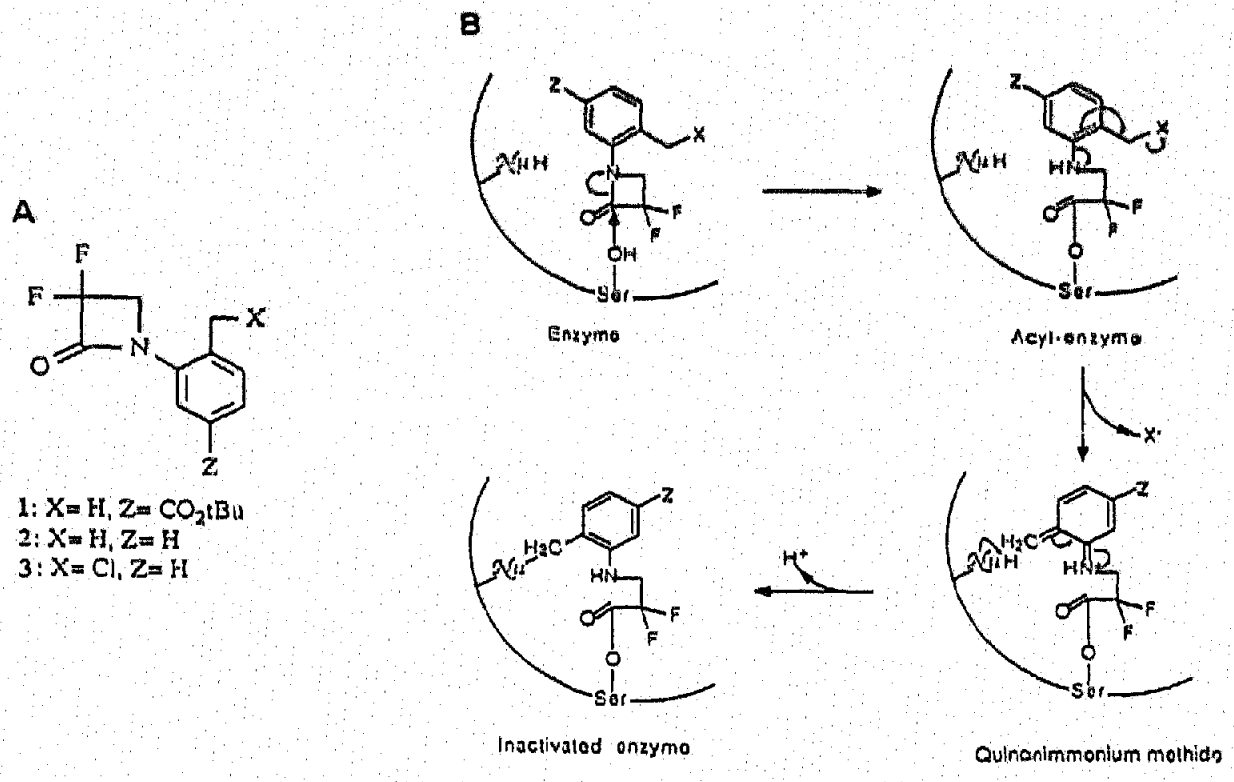

Fig. 1. Structure of compounds 1–3 (A) and postulated mechanism for the inhibition (B).

Kabi Vitrum (S-2251 for plasmin and S-2238 for thrombin). PNA and NPGB were from Sigma; Z-Ala-Ala-Pro-Aala-Ala-ONp was a kind gift from Dr J. Bieth (Université Louis Pasteur, F-67400 Illkirch). Active-site titrations were carried out with Z-Ala-Ala-Pro-Aala-Ala-ONp for both elastases [14], PNA for chymotrypsin [15] and NPGB for trypsin, plasmin and thrombin [16]. *N*-(2-Methylphenyl) 3,3-difluoro-azetidin-2-one **2** was obtained from 3-bromo-2,2-difluoro-propanoyl chloride and *o*-toluidine according to the procedure described for the preparation of *N*-(3-*tert*butyloxycarbonyl-6-methylphenyl) 3,3-difluoro-azetidin-2-one **1** [11]. *N*-(2-chloromethylphenyl) 3,3-difluoro-azetidin-2-one **3** was prepared in a similar way starting from 2-(*tert*butyldimethylsilyloxymethyl)-aniline [17]. The amide thus obtained was cyclized to azetidin-2-one, the *tert*butyldimethylsilyloxy group of which was cleaved by aqueous HF in CH_3CN. Finally, treatment of the resulting *N*-(2-hydroxymethylphenyl) 3,3-difluoroazetidin-2-one by $SOCl_2$/DMF provided the azetidin-2-one **3** (AA 231-1). Details of the synthesis will be reported elsewhere. All kinetic studies were performed using a Lambda 5 Perkin Elmer spectrophotometer equipped with a thermostatted cell holder. The pH was measured before and after each run using a pH-stat Radiometer type TT1c equipped with a glass B electrode.

2.2. *Enzymic activity assays*

The amidolytic activities of PPE, HLE, chymotrypsin, plasmin and thrombin were determined at 37°C towards the appropriate chromogenic substrate. The total assay volume was 1 ml in the following buffers: 0.1 M Tris (pH 8.0) for PPE; 0.1 M Tris (pH 8.0), 0.01% Brij 35 for HLE; 0.025 M sodium phosphate (pH 7.5), 0.1 M KCl for chymotrypsin; 0.05 M Tris (pH 7.5), 0.01 M $CaCl_2$ for trypsin; 0.1 M sodium phosphate (pH 7.3), 25% (v/v) glycerol for plasmin, and 0.05 M Tris (pH 8.0), 0.04 M NaCl, 0.1% (v/v) PEG 6000 for thrombin. The concentration of the substrate was 100 μM (80 μM for thrombin) and the enzyme concentrations were: 0.22 μM (PPE), 27 nM (HLE), 0.2 μM (chymotrypsin, plasmin), 60 μM (trypsin) and 0.03 μM (thrombin).

2.3. *Enzymic hydrolysis of azetidinones* **1** *and* **2**

PPE and HLE activities towards compounds **1** and **2** were measured spectrophotometrically at pH 8.0 and 37°C in 0.1 M Tris buffer ($[PPE]_0$ = 3.1 and 3.63 μM, respectively) and 0.1 M Tris buffer (pH 8.0), 0.01% (v/v) Brij 35 ($[HLE]_0$ = 2.4 and 1 μM, respectively). At least 7 different concentrations of the azetidinones were used, within the following ranges: 15–100 μM (**1**, PPE), 20–73 μM (**1**, HLE), 71–471 μM (**2**, PPE) and 140–700 μM (**2**, HLE). The ratio k_{cat}/K_M was determined from least-squares analysis of initial rates against initial substrate concentrations. The concentrations of the end products were determined using the molar absorption coefficients: 1600 M^{-1} cm^{-1} at 320 nm for **1** and 920 M^{-1} cm^{-1} at 300 nm for **2**. After 90 min incubation at pH 8.0 (0.1 M Tris) and 37°C, a mixture containing 4% (v/v) DMSO, PPE (6 μM) and **1** (500 μM) or **2** (250 μM) was filtered on Centricon 10 microconcentrator. The product of the enzymic digestion was characterized by isocratic HPLC (Waters model 510) on a 5 μm C_8 Nucleosil column (SFCC, 250 × 20 mm) using 30–70% (v/v) isopropanol/water as eluent. The flow rate was 0.75 ml/min and the effluent was monitored at 260 nm. The starting azetidinones **1** and **2** were chromographed under the same experimental conditions.

2.4. *Elastase inactivation procedure*

Two different methods were used to study the action of reagent **3**. The first was the classical method of preincubating enzyme and inhibitor and after a fixed period, or at intervals of time, small aliquots (10 μl) were withdrawn. HLE (1.35 μM) and PPE (3.6 μM) were treated at 37°C and pH 8.0 (0.1 M Tris, 5% (v/v) DMSO and for HLE, 0.01% Brij 35) with a molar excess of inhibitor over enzyme ranging from 10 to 100 (HLE) and 2 to 10 (PPE). The reaction was stopped either by dilution of aliquots (factor of 100) or by dilution followed by filtration at 4°C on Centricon 10 microconcentrators (Amicon) and washing with buffer. The samples were assayed for residual enzymic activity by addition to 1 ml of the appropriate standard assay mixture (see above). Enzyme activity was expressed

relatively to an elastase blank containing 5% DMSO but without inhibitor to correct for the spontaneous inactivation of the enzyme. To determine the reversible or irreversible character of the inhibition, filtered aliquots of the reaction mixtures were stored at 4°C for 18 h before checking remaining activity. In other experiments, after filtration, the enzymes were treated for 30 min with 1 M NH_2OH at pH 8.6, 25°C, and filtered again before determination of activity. Some pseudo-first-order constants k_{obs} for the inactivation of PPE and HLE by 3 were obtained from least-squares analysis of semilog plots of the percentage of remaining activity against time. This incubation method was also used to evaluate the effect of 1 and 2 on both elastases (using a ratio $[I]_0/[E]_0$ of 300) and of 3 on the activity of other serine proteases in the following conditions: trypsin (14 μM), chymotrypsin (3.8 μM), plasmin (87 nM) and thrombin (94 nM); $[I]_0/[E]_0 = 100$, pH 7.5 (8.0 for thrombin), 37°C, 15 min incubation. The second method (progress curve analysis) was used to determine more accurately the kinetic parameters of the inactivation of HLE (27 nM) and PPE (220 nM) by the inhibitor 3 (22.3–223 μM and 17.2–149 μM, respectively) at 37°C, pH 8.0, 3.5 and 3% (v/v) DMSO, respectively, in the presence of chromogenic substrate S. The general reaction of a serine protease with a mechanism-based inactivator is considered to proceed according to eq. 1 [18], where E is the enzyme, I the inhibitor, I' the hydrolyzed inhibitor, E–I the acyl enzyme and E–I the inactivated enzyme.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} E\text{–}I \xrightarrow{k_3} E\text{–}I \qquad \text{(eq. 1)}$$
$$\downarrow k_{cat}$$
$$E + I'$$

EI and E–I are not observed independently and k_2 and k_3 may be combined to give the simplified expression shown in eq. 2 (line 1) where E*I is a steady state enzyme–inhibitor complex formed prior to k_2 (in this simplified scheme, k_{cat} is neglected). Eq. 2 describes the competition between I and S. The rate of change in absorbance v at 405 nm due to the hydrolysis of the appropriate substrate (100 μM) as compared to a cuvette containing the same amount of inhibitor and substrate in buffer was directly obtained from a computer-assisted spectrophotometer and analyzed according to Hart and O'Brien [19].

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E^*I \xrightarrow{k_{inact}} E\text{–}I \qquad \text{(eq. 2)}$$
$$S \underset{K_m}{\rightleftharpoons} ES \xrightarrow{k_+} E + P$$

A plot of ln v versus time gave a straight line with a slope $-\pi$. According to eq. 3, a plot of $1/\pi$ versus $1/[I]$ $(1-\alpha)$, with $\alpha = [S]/(K_m + [S])$, analyzed by statistical treatment of Wilkinson [20] gave a straight line with an x intercept of $1/K_I$ and y intercept of $1/k_{inact}$.

$$1/\pi = K_I/k_{inact}\,[I]\,(1-\alpha) + 1/k_{inact} \qquad \text{(eq. 3)}$$

Standard errors for K_I and k_{inact} were obtained as described by Wilkinson. The values of K_m (2.6 and 0.14 mM for PPE and HLE, respectively) were determined under the following experimental conditions using the appropriate buffer: [PPE] = 114 nM and [Suc-Ala_3-NA]$_0$ = 0.03–8 mM; [HLE] = 13.5 nM and [MeO-Suc-Ala_2-Pro-Val-NA] = 0.05–1 mM.

2.5. *Determination of the partition ratio*

Experiments were carried out as described above using the preincubation method, with concentrations that would not totally inactivate enzyme. After 25 min incubation at 25°C, the mixtures were stirred gently at 4°C for 16–17 h before determining residual activity.

2.6. *Inhibition of [^{3}H]elastin solubilization*

[^{3}H]Elastin was prepared as described before [12]. Three series of samples of 800 μg (4.44 kBq) insoluble tritiated elastin were incubated at 37°C with PPE (83 nM). At times 0, 20 and 60 min after the beginning of this reaction, each series of samples was treated with inhibitor at various final concentrations ranging from 5 to 48 μM (total volume of 400 μl). One sample in each series was taken hourly for centrifugation and the supernatant was counted for radioactivity.

3. RESULTS AND DISCUSSION

3.1. *Enzymic hydrolysis of azetidinones 1 and 2 catalyzed by PPE and HLE*

Azetidinones 1 and 2 are more efficiently hydrolyzed in the presence of HLE (k_{cat}/K_m = 1125 M^{-1} s^{-1} for 1 and 1100 M^{-1} s^{-1} for 2) than PPE (k_{cat}/K_m = 340 M^{-1} s^{-1} for 1 and 500 M^{-1} s^{-1} for 2). By scanning the reaction mixture at regular intervals in the range 190–400 nm, the spectra showed new absorbance peaks at 319 (1) and 285 (2) nm characteristic of an aniline moiety. In each case, isocratic reversed-phase HPLC of the PPE reaction mixtures showed only the formation of one hydrolysis product having retention times of 11.6 min for 1 and 9 min for 2 compared with 26.6 and 12 min for the starting azetidinones, respectively (Fig. 2).

3.2. *Inactivation of HLE and PPE by azetidinone 3*

Exposure of PPE and HLE to reagent 3 resulted in a time- and concentration-dependent inhibition. For both enzymes, the inhibition was irreversible and no significant recovery of enzymic activity was observed after elimination of the reagent. For example, the percentages of remaining activity for PPE treated during 20 min by a 20-fold molar excess of reagent were 0.1% and 0.6% before and after filtration followed by incubation at 4°C. To analyze the kinetics of PPE and HLE inactivation by 3, we followed the progress curve of substrate hydrolysis run in a competitive fashion in the presence of a concentration of inhibitor which causes first-order enzyme inactivation (Fig. 3A, B). The apparent dissociation constant of the enzyme–inhibitor complex K_I and the first-order constant at infinite concentration of inhibitor k_{inact} were obtained by analysis of changing slopes of the progress curves with time as described in section 2 (Fig. 3C). The

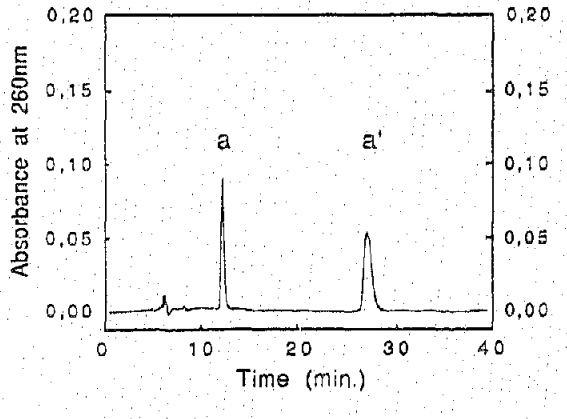

Fig. 2. HPLC chromatogram of PPE hydrolysate of 1 (see section 2.4). Peak a = product of hydrolysis of 1; peak a' = 1.

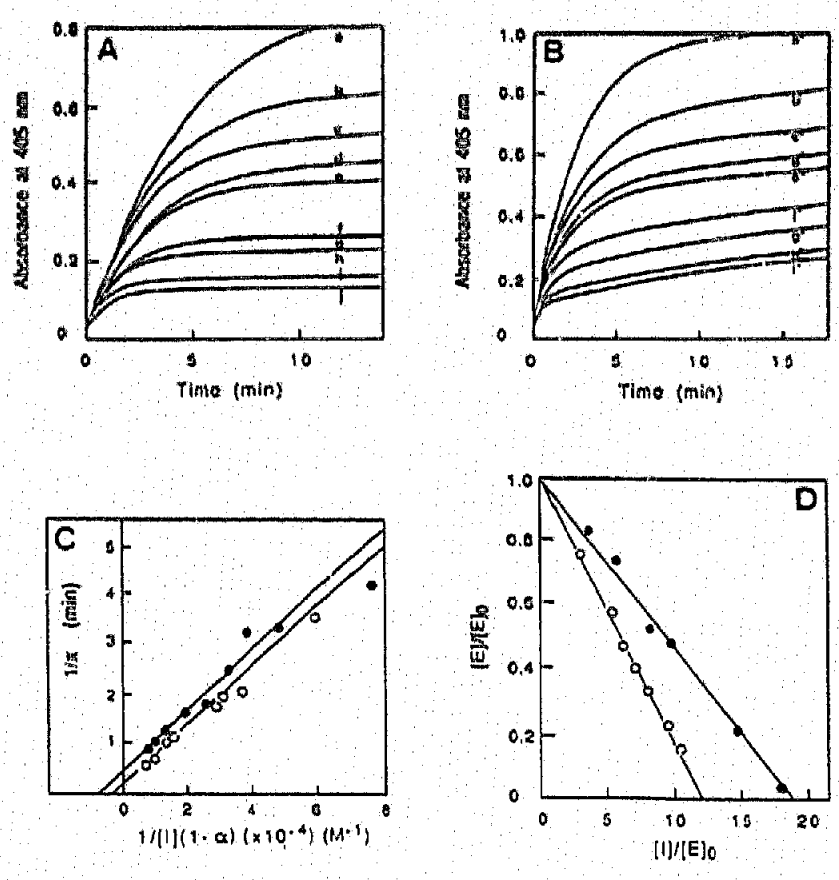

Fig. 3. Inactivation of HLE and PPE with reagent 3. (A and B) Time course of hydrolysis of MeO-Suc-Ala$_2$-Pro-Val-NA (100 μM) by HLE (27 nM) (A) and of Suc-Ala$_3$-NA (100 μM) by PPE (220 nM) (B) in the absence (a,a') and in the presence of various concentrations (in μM) of 3: (b) 22.3; (c) 35.7; (d) 44.7; (f) 53.6; (g) 89.3; (h) 134; (i) 179; (j) 223; (b') 17.2; (c') 28; (d') 33.6; (e') 34.5; (f') 67.2; (g') 78.4; (h') 112; and (i') 149, at pH 8.0 and 37°C. (C) Determination of K_I and k_{inact}: (●) HLE; (○) PPE. (D) Plot of the fraction of remaining activity (determined using the preincubation method) versus $[I]/[E]_0$: (●) HLE; (○) PPE.

values obtained for K_I were $(2.7 \pm 0.5) \times 10^{-4}$ M (PPE) and $(1.2 \pm 0.1) \times 10^{-4}$ M (HLE), and for k_{inact} 0.08 ± 0.01 s^{-1} (PPE) and 0.035 ± 0.002 s^{-1} (HLE) at pH 8.0 and 37°C.

3.3. *Characteristics of the inactivation process*

The inhibitor competes with the substrate (see progress curves); increasing amounts of substrate at fixed concentration of inhibitor protect the enzyme indicating that the inhibition occurs at the active site. In the presence of a large molar excess of azetidinones over enzyme (300), compounds **1** and **2** failed to inactivate both elastases, suggesting that the inactivation observed for their functionalized analogue **3** is not due to the mere formation of a stable acyl-enzyme. This was confirmed by the absence of reaction (<1%) observed when the inhibited enzymes were treated by buffered hydroxylamine, in agreement with an irreversible chemical modification of an enzyme amino acid residue different from active serine. The partition ratio which represents the average number of enzyme 'turnovers per inactivation' k_{cat}/k_{inact} (eq. 4) was evaluated by the titration method [18] (Fig. 3D).

$$E + I \overset{K_I}{\rightleftharpoons} E^*I \xrightarrow{k_{inact}} E - I \qquad \text{(eq. 4)}$$
$$\downarrow k_{cat}$$
$$E + I'$$

The linear plot of $[E]/[E]_0$ at infinite time versus $[I]_0/[E]_0$ is described by eq. 5 [21] and its intercept with the x axis minus 1 [18] gives partition ratios of 11 and 18 for PPE and HLE, respectively.

$$[I]_0/[E]_0 = \frac{k_{inact} + k_{cat}}{k_{inact}} (1 - [E]/[E]_0) \qquad \text{(eq. 5)}$$

A catalytic step prior to inactivation by **3** as proposed in the postulated mechanism (Fig. 1) is supported by the observed hydrolysis of the unfunctionalized azetidinones **1** and **2** suggesting an attack of the β-lactam carbonyl group by active serine. Several factors contribute to the high reactivity of azetidinone **3**: the ring strain, the polarization of the β-lactam carbonyl by the *gem*-difluoro group which, through its inductive effect, should decrease the pK of the conjugated acid of the aniline leaving group [11,22]. The formation of the acyl-enzyme simultaneously unmasks a benzyl halide possessing a strong electron-releasing amino substi-

tuent in the *ortho* position. A fast elimination of X^- should give a very electrophilic quinonimmonium methide ion [13]. Owing to the cyclic nature of the inhibitor, this electrophile is tethered by a covalent linkage in the active site during the lifetime of the acyl enzyme. Then, by reaction of the electrophile with another active site nucleophile, an inactivated doubly-linked enzyme may result as reported in other cases [23]. The IC_{50} value (concentration of 3 that gives 50% inhibition 2 min after mixing enzyme and inhibitor) was found equal to 0.01 mg/ml (a value of 0.01 μg/ml was found for other monocyclic β-lactams [10]). HLE is inhibited less efficiently (factor of 3.4) by 3 than by a bromo enol lactone [24] but this reagent also inhibits chymotrypsin. Interestingly, the inhibitor 3 does not show significant efficiency to inhibit chymotrypsin, trypsin, plasmin and thrombin ($[I]_0/[E]_0 = 100$).

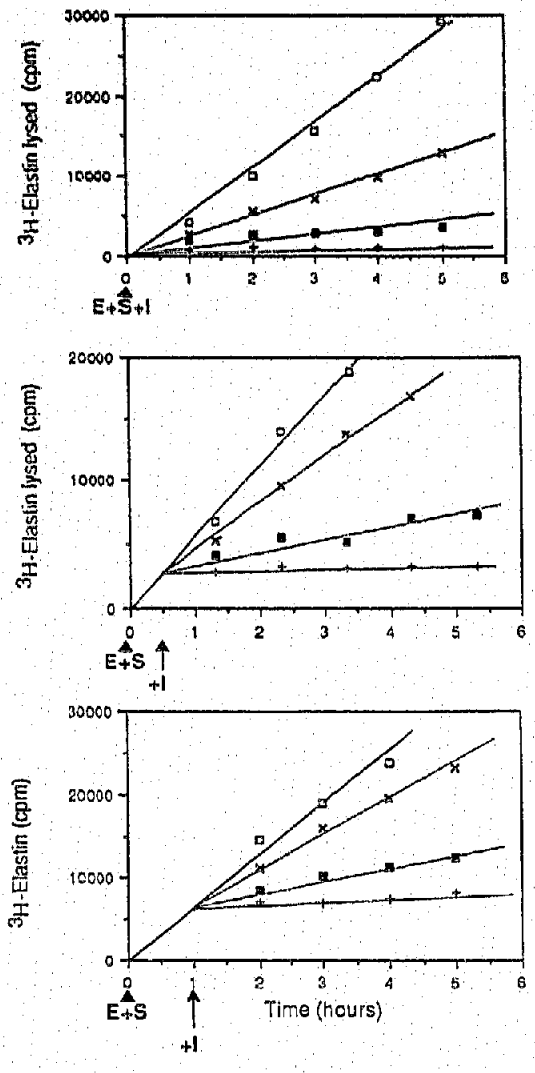

Fig. 4. [^{3}H]Elastin solubilization (800 μg) by PPE (83 nM) in the absence (□) and in the presence of reagent 3: (X) 5 μM; (■) 16 μM; (+) 48 μM. The inhibitor was added after various times of incubation of PPE with elastin: (A) 0, (B) 20 and (C) 60 min.

3.4. *Inhibition of elastin solubilization*

The solubilization of [^{3}H]elastin by PPE is inhibited by 3 as shown in Fig. 4. The values found for IC_{50} determined from a plot of v/v_0 versus molar excess $[I]_0/[E]$ (v and v_0 are the lysis rates with and without inhibitor, respectively) were only decreased by a factor of 1.6 and 2.3, respectively, when elastase and elastin were preincubated 20 and 60 min before treatment by the inhibitor. This interesting feature may be of therapeutical value.

Acknowledgement: We thank C. Favreau for technical assistance.

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