Kinetics of the inhibition of human pancreatic elastase by recombinant eglin c

Influence of elastin

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Recombinant eglin c is a potent reversible inhibitor of human pancreatic elastase. At pH 7.4 and 25 °C, $k_{\text{ass.}} = 7.3 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{\text{diss.}} = 2.7 \times 10^{-4} \text{ s}^{-1}$ and $K_i = 3.7 \times 10^{-10} \text{ M}$. Stopped-flow kinetics indicate that the formation of the stable enzyme–inhibitor complex is not preceded by a fast pre-equilibrium complex or that the latter has a dissociation constant greater than 0.3 μM . The elastase–eglin c complex is much less stable at pH 5.0 and 25 °C, where $k_{\text{diss.}} = 1.1 \times 10^{-2} \text{ s}^{-1}$ and $K_i = 7.3 \times 10^{-8} \text{ M}$. At pH 7.4 the activation energy for $k_{\text{ass.}}$ is 43.9 $\text{kJ} \cdot \text{mol}^{-1}$ (10.5 $\text{kcal} \cdot \text{mol}^{-1}$). The $k_{\text{ass.}}$ increases between pH 5.0 and 8.0 and remains essentially constant up to pH 9.0. This pH-dependence could not be described by a simple ionization curve. Both α_2 -macroglobulin and α_1 -proteinase inhibitor are able to dissociate the elastase–eglin c complex, as evidenced by measurement of the enzymic activity of α_2 -macroglobulin-bound elastase or by polyacrylamide-gel electrophoresis of mixtures of α_1 -proteinase inhibitor and elastase–eglin c complex. The rough estimate of $k_{\text{diss.}}$ obtained with the α_2 -macroglobulin dissociation experiment ($1.6 \times 10^{-4} \text{ s}^{-1}$) was of the same order of magnitude as the constant measured with the progress curve method. Eglin c strongly inhibits the solubilization of human aorta elastin by human pancreatic elastase. The extent of inhibition is the same whether elastase is added to a suspension of elastin and eglin c or whether elastase is preincubated with elastin for 3 min before addition of eglin c. However, the efficiency of the inhibitor sharply decreases if elastase is reacted with elastin for more prolonged periods.

INTRODUCTION

Eglin c is an 8.1 kDa protein proteinase inhibitor first isolated from the leech *Hirudo medicinalis* [1] and now produced by genetic engineering as an *N*-acetyl derivative [2]. This inhibitor is composed of a single polypeptide chain of 70 amino acid residues. It is extremely stable despite the lack of disulphide bridges [3]. Eglin c belongs to the potato inhibitor I family of serine-proteinase inhibitors. It potently inhibits chymotrypsin, subtilisin, neutrophil elastase and cathepsin G, forms loose complexes with bovine pancreatic trypsin and pig pancreatic elastase, and does not inhibit plasmin, thrombin and kallikrein [1,4,5]. The effect of eglin c on human pancreatic elastase has not been reported. This enzyme is a 25 kDa serine proteinase synthesized and stored in the pancreas as a proenzyme. It is composed of a single polypeptide chain of 241 amino acid residues, as determined by sequence analysis of the cloned mRNA [6]. Elastase is able to solubilize fibrous elastin at a high rate [7–9]. Its postulated pathogenic role in acute pancreatitis [10–12] and atherosclerosis [13] is probably related to its elastolytic properties. The bovine pancreatic trypsin inhibitor (aprotinin), which is sometimes used in pancreatitis, does not inhibit elastase [8]. It was therefore of interest to see whether recombinant eglin c inhibits elastase and whether the inhibition also takes place in the presence of its natural substrate, elastin.

EXPERIMENTAL

Materials

Published procedures were used to purify human pancreatic elastase [7], human plasma α_1 -proteinase inhibitor [14], α_2 -macroglobulin [15] and human aorta elastin [16]. Recombinant

eglin c [2] was obtained from CIBA–GEIGY (Basel, Switzerland) through the courtesy of Dr. H. P. Schnebli.

Glutaryl-Ala₂-Pro-Leu-*p*-nitroanilide and 3-carboxypropionyl-Ala₂-Pro-Phe-*p*-nitroanilide [17] were from the Peptide Research Institute (Osaka, Japan) and Bachem (Bubendorf, Switzerland) respectively. Stock solutions of these substrates were made in dimethylformamide unless otherwise stated.

Buffers

Most experiments were done in a buffer solution containing 50 mM-Hepes and 100 mM-NaCl, pH 7.4 (buffer A). When the synthetic substrates were used, the buffer also contained 2% (v/v) dimethylformamide unless otherwise stated. When the pH was varied, the following buffers were used: 50 mM-acetate (pH 5.0 and 5.5), 50 mM-Mes (pH 6.0 and 6.5), 50 mM-Hepes (pH 7.0 and 7.4) and 50 mM-Tris (pH 8.0, 8.5 and 9.0). All these buffers also contained 100 mM-NaCl.

Active-site titrations

Eglin c and α_1 -proteinase inhibitor were titrated with active-site titrated human neutrophil elastase [5,14]. Pig pancreatic elastase was used to titrate α_2 -macroglobulin [18]. To titrate elastase, 1 μM enzyme was reacted with increasing concentrations of titrated eglin in Buffer A. After 5 min at 25 °C, the residual enzyme activities were measured with 0.35 mM succinyl-Ala₂-Pro-Phe-*p*-nitroanilide. Under these favourable titration conditions ([elastase] = 3000 K_i , see below) a straight inhibition curve was obtained that allowed the elastase titre to be calculated.

Kinetics of the inhibition of elastase by eglin c

Progress curves for the inhibition of elastase by eglin c in the presence of glutaryl-Ala₂-Pro-Leu-*p*-nitroanilide were recorded

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at 410 nm. When the reaction was followed for more than 2 min, a Cary 2200 spectrophotometer with a thermostated cell holder was used. Absorbances were continuously measured, digitized, and stored in an IBM PS/2 model 30 microcomputer. Progress curves were composed of 2300 (absorbance, time) pairs.

Faster reactions were monitored with a High-Tech model SF/PQ53 stopped-flow apparatus with an SU-40 amplifier and a water circulation. Each syringe was filled with a buffered solution of either elastase or eglin c + substrate; 100 μ l of solution from each syringe was used per run, and an average of five runs was performed for each determination of the constant. The continuously measured absorbances were digitized and stored in an HP-9000 model 300 microcomputer. Here the progress curves were composed of 400 (absorbance, time) pairs.

All experiments were done under pseudo-first-order conditions with a 10-fold molar ratio of eglin c to elastase. Less than 0.2 % of substrate was hydrolysed at the end of the reactions.

Dissociation of the elastase–eglin c complex by α_2 -macroglobulin

Elastase (100 nM) was reacted for 10 min at 25 °C with 100 nM-eglin c. At time zero, 1 vol. of complex was diluted with 9 vol. of 110 nM- α_2 -macroglobulin (all proteins were dissolved in buffer A containing 10 μ g of BSA/ml). After selected periods 950 μ l of this mixture were removed and poured into a spectrophotometer cuvette containing 50 μ l of 24 mM-glutaryl-Ala₂-Pro-Leu-*p*-nitroanilide dissolved in the above buffer. The absorbance at 410 nm was then recorded to measure the activity of α_2 -macroglobulin-bound elastase. Separate experiments showed that the specific activity of the latter was 55 % that of free elastase. The controls shown in Fig. 5 were incubated and assayed in a similar fashion.

Determination of k_{cat} and K_m

The kinetic parameters for the elastase-catalysed hydrolysis of glutaryl-Ala₂-Pro-Leu-*p*-nitroanilide were measured at pH values between 5.0 and 9.0 (see buffers above) and 25 °C. The elastase concentration ranged from 250 nM (pH 5.0) to 50 nM (pH 7.0 and above). Initial velocities were measured at seven to nine substrate concentrations. Double-reciprocal plots showed that the kinetics adhered to the Michaelis–Menten model and provided the initial estimates of k_{cat} and K_m . The refined values of these parameters and their confidence intervals were calculated by non-linear least-squares analysis. The errors on k_{cat} and K_m were less than 4 % and 12 % respectively.

All other technical details are given in the legends to the Figures.

RESULTS

Kinetics of elastase–eglin c complex formation and dissociation in the presence of substrate

Preliminary experiments showed that eglin c was a tight-binding elastase inhibitor. For instance, more than 80 % inhibition of the activity on glutaryl-Ala₂-Pro-Leu-*p*-nitroanilide

Fig. 1. Progress curves for the inhibition of elastase by eglin c at pH 7.4 and 25 °C

Absorbance was recorded for reaction solutions containing 1 nM-elastase, 10 nM-eglin c and 10.4 mM-glutaryl-Ala₂-Pro-Leu-*p*-nitroanilide. The reaction was initiated by the addition of 10 μ l of elastase to 990 μ l of eglin c + substrate (a) or 10 μ l of elastase–eglin c complex to 990 μ l of substrate (b).

was observed upon treating 10 nM-elastase with 10 nM-eglin c for 10 min at pH 7.4 and 25 °C. Furthermore, when 2 μ M-enzyme–inhibitor complex was diluted 100-fold into a 20 mM solution of the above substrate, the product–time curve became concave upwards, suggesting that the inhibition is reversible and competitive.

To determine the kinetic constants describing the elastase–eglin c interaction, we recorded the formation of product following either addition of enzyme to a mixture of inhibitor and substrate (Fig. 1a) or addition of enzyme–inhibitor complex to substrate (Fig. 1b). The biphasic progress curves were analysed assuming that eglin c (I) and substrate (S) compete for the binding of elastase (E) as shown in Scheme 1, where $k_{ass.}$ is the second-order rate constant for the formation of the EI complex and $k_{diss.}$ is the first-order rate constant for the decomposition of this complex. If neither the inhibitor nor the substrate is depleted to a significant extent during the progress of the reaction (i.e. if $[I_0] \gg [E_0]$ and $[P] \ll [S_0]$), biphasic progress curves such as those shown in Fig. 1 can be described by the following integrated equation [19]:

$$[P] = v_s t + (v_0 - v_s)(1 - e^{-kt})/k \quad (1)$$

where $[P]$ is the product concentration, v_0 is the velocity at $t = 0$,

Scheme 1.

Table 1. Rate and equilibrium constants describing the elastase–eglin c interaction at 25 °C

The errors on the parameters are less than 15 %. K_I was calculated from $k_{diss.}/k_{ass.}$

pH	$k_{ass.}$ ($M^{-1} \cdot s^{-1}$)	$k_{diss.}$ (s^{-1})	K_I (M)
5.0	1.5×10^5	1.1×10^{-2}	7.3×10^{-8}
5.5	2.6×10^5	5.5×10^{-3}	2.1×10^{-8}
7.4	7.3×10^5	2.7×10^{-4}	3.7×10^{-10}

Scheme 2.

v_s is the steady-state velocity and k is the apparent first-order rate constant for the approach to steady state.

The progress curves shown in Figs. 1(a) and 1(b) were fitted to eqn. (1) by non-linear least-squares analysis to obtain the best estimates of v_0 , v_s and k . Very similar values of v_s and k were obtained for the curves of Figs. 1(a) and 1(b). This indicates that v_s is a true steady-state velocity and that eqn. (1) properly describes the progress curves.

The following relationships were used to calculate $k_{\text{ass.}}$ and $k_{\text{diss.}}$ from v_0 , v_s and k [19]:

$$k = k_{\text{ass.}}[I_0]/(1 + [S_0]/K_m) + k_{\text{diss.}} \quad (2)$$

$$k_{\text{diss.}} = k \cdot v_s/v_0 \quad (3)$$

The experiments illustrated in Fig. 1 were repeated several times using identical concentrations but different solutions of elastase, eglin c and substrate. These measurements gave very close kinetic constants whose average values are shown in Table 1. It can be seen that at neutral pH eglin c is a tight-binding inhibitor of elastase.

It is likely that the stable EI complex does not form directly from E and I as illustrated in Scheme 1 but that the reaction proceeds through a fast pre-equilibrium as shown in Scheme 2.

The apparent first-order rate constant k of eqn. (1) is now given by the following equation [20]:

$$k = k_{+i}[I_0]/([I_0] + K_i^*(1 + [S_0]/K_m)) + k_{-i} \quad (4)$$

In an attempt to detect EI*, we measured k using a concentration of inhibitor 40-fold higher than that employed in Fig. 1. The rapid progress curves were recorded with a stopped-flow apparatus (Fig. 2). This experiment was repeated with various

Fig. 2. Progress curve for the inhibition of elastase by eglin at pH 7.4 and 25 °C

The reaction medium contained 40 nM-elastase, 400 nM-eglin c and 2 mM-glutaryl-Ala₂-Pro-Leu-*p*-nitroanilide. The reaction was initiated by mixing equal volumes of enzyme and inhibitor + substrate with a stopped-flow apparatus. The line crossing the stopped-flow trace is the theoretical progress curve corresponding to best fit. This experiment was repeated with three other substrate concentrations. Inset: substrate-dependency of the apparent rate constant k .

substrate concentrations and k was plotted as a function of $1/(1 + [S_0]/K_m)$ (Fig. 2 inset). The linearity of this plot indicates that Scheme 1 is obeyed (see eqn. 2). Scheme 2 predicts a hyperbolic dependence of k upon $1/(1 + [S_0]/K_m)$ (see eqn. 4). The pre-equilibrium, if any, is therefore not seen kinetically in these experiments. The decrease of k with $[S_0]$ also provides evidence for the assumed competitive nature of the inhibition.

The slope of the line in Fig. 2 inset is equal to $k_{\text{ass.}}[I_0]$ (see eqn. 2). The $k_{\text{ass.}}$ calculated from this slope ($7.5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$) is in excellent agreement with that measured at low inhibitor concentration (Table 1). The dissociation rate constant $k_{\text{diss.}}$ could not be calculated from Fig. 2 inset because the line passed through the origin (i.e. $k_{\text{ass.}}[I_0] \gg k_{\text{diss.}}$).

pH- and temperature-dependency of the inhibition

The effect of pH and temperature on the rate of inhibition of elastase by eglin c was studied with the stopped-flow apparatus as indicated in the legends to Figs. 3 and 4. Owing to the high inhibitor concentration used (1 μM), most progress curves reached a plateau, indicating that $k_{\text{diss.}}$ was much smaller than $k_{\text{ass.}}[I_0]/(1 + [S_0]/K_m)$ (see eqn. 2) and could therefore not be measured. At pH 5.0 and 5.5, however, biphasic progress curves were recorded so that both constants could be determined. These are reported in Table 1. It can be seen that $k_{\text{diss.}}$ increases 40-fold between pH 7.4 and 5.0. This increase is accompanied by a 5-fold decrease in $k_{\text{ass.}}$ and a resultant 200-fold increase in K_i . The elastase-eglin c affinity is thus strongly pH-dependent. Fig. 3(a) shows that $k_{\text{ass.}}$ increases almost linearly between pH 5.0 and pH 8.0 and remains essentially constant up to pH 9.0. The pH-dependence of $k_{\text{ass.}}$ could thus not be accounted for by the ionization of a single residue on the enzyme or the inhibitor. In contrast, $k_{\text{cat.}}/K_m$, the second-order rate constant for the reaction of the enzyme with the substrate, exhibits a bell-shaped pH-dependence, suggesting the participation of two ionizable groups

Fig. 3. pH-dependency of the second-order rate constant, $k_{\text{ass.}}$, for the inhibition of elastase by eglin c (a) and of the second-order rate constant, $k_{\text{cat.}}/K_m$, for the elastase-substrate interaction (b)

Both constants were measured at 25 °C. The nature of the buffers and the measurement of $k_{\text{cat.}}/K_m$ are given in the Experimental section. Progress curves used to measure $k_{\text{ass.}}$ were generated using a stopped-flow apparatus. One syringe contained 0.2 μM -elastase while the other was filled with a mixture of 2 μM -eglin c and 5 mM-glutaryl-Ala₂-Pro-Leu-*p*-nitroanilide (final concentrations: $[E_0] = 0.1 \mu\text{M}$, $[I_0] = 1 \mu\text{M}$, $[S_0] = 2.5 \text{ mM} = 1.9 K_m$).

Fig. 4. Arrhenius plot for the second-order rate constant, k_{ass} , for the inhibition of elastase by eglin c at pH 7.4

The k_{ass} values were determined with a stopped-flow apparatus as indicated in the legend to Fig. 3. The k_{ass} data were expressed in dimensionless numbers as suggested by Keleti [23]: relative k_{ass} = k_{ass} at a given temperature divided by k_{ass} at 5 °C.

with acidic and basic pK values (pK_1 , pK_2). The data were fitted by non-linear regression analysis to the following equation [21]:

$$k_{\text{cat.}}/K_m = \frac{(k_{\text{cat.}}/K_m)_{(\text{lim})}}{1 + [\text{H}^+]/K_1 + K_2/[\text{H}^+]} \quad (5)$$

where $(k_{\text{cat.}}/K_m)_{(\text{lim})}$ is the limiting value of $k_{\text{cat.}}/K_m$. The curve corresponding to the best fit is shown in fig. 3(b) and corresponds to $pK_1 = 5.9 \pm 0.2$ and $pK_2 = 9.3 \pm 0.3$. These are classical pK values for a chymotrypsin-like enzyme with pK_1 and pK_2 corresponding to His-57 of the active centre and the α -NH₂ group of the *N*-terminal amino acid residue respectively [22].

Fig. 4 depicts the effect of temperature on k_{ass} . An energy of activation of $43.9 \text{ kJ} \cdot \text{mol}^{-1}$ ($10.5 \text{ kcal} \cdot \text{mol}^{-1}$) was calculated from the Arrhenius plot.

Dissociation of the elastase–eglin c complex by α_2 -macroglobulin and α_1 -proteinase inhibitor

To confirm the reversibility of the elastase + eglin c association and the order of magnitude of k_{diss} by substrate-independent methods, we used two irreversible ligands (L) of elastase as dissociating agents, namely α_2 -macroglobulin and α_1 -proteinase inhibitor [9] (see Scheme 3). The former is a convenient dissociating agent because $k_L \gg k_{\text{ass}}$ (k_L is $6.4 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$) and EL is enzymically active on synthetic substrates [9]. In the experiment shown in Fig. 5 we used a 10-fold molar excess of α_2 -macroglobulin over the elastase–eglin c complex to ascertain that k_{diss} was the rate-limiting step for the formation of EL. It can be seen that α_2 -macroglobulin is able to take up the elastase bound to eglin c in a time-dependent manner. Full reversal of inhibition took about 6 h. This experiment was complicated by the observ-

Fig. 5. Dissociation of the elastase–eglin c complex by α_2 -macroglobulin at pH 7.4 and 25 °C

●, Time-dependent appearance of α_2 -macroglobulin–elastase activity after mixing 10 nM-elastase–eglin c complex with 100 nM- α_2 -macroglobulin. Control experiments: ○, 10 nM-elastase + 100 nM- α_2 -macroglobulin; △, 10 nM-elastase + 100 nM- α_2 -macroglobulin + 10 nM-eglin c. Elastase and α_2 -macroglobulin were preincubated for 5 min at 25 °C.

Fig. 6. Dissociation of the elastase–eglin c complex by α_1 -proteinase inhibitor at pH 7.4 and 37 °C

The complex ($14 \mu\text{M}$) was incubated with $30 \mu\text{M}$ - α_1 -proteinase inhibitor in buffer A, and samples from this mixture were withdrawn, electrophoresed at pH 8.8 and stained with Coomassie Brilliant Blue using the Phastsystem apparatus (Pharmacia) and an 8–25% polyacrylamide gradient gel without SDS. Lane 1: $14 \mu\text{M}$ -elastase (HPE) + $30 \mu\text{M}$ - α_1 -proteinase inhibitor ($\alpha_1\text{PI}$). Lane 2: $30 \mu\text{M}$ - α_1 -proteinase inhibitor. Lane 3: $14 \mu\text{M}$ -elastase–eglin c complex. Lanes 4 and 5: $14 \mu\text{M}$ -elastase–eglin c complex incubated with $30 \mu\text{M}$ - α_1 -proteinase inhibitor for 22 min (lane 4) or 90 min (lane 5).

ations that the activity of the α_2 -macroglobulin–elastase complex increased with time and that eglin c was able partially to inhibit α_2 -macroglobulin-bound elastase. These effects precluded a precise calculation of k_{diss} . The $t_{1/2}$ of the dissociation processes (72 min) gave a rough estimate of k_{diss} of $1.6 \times 10^{-4} \text{ s}^{-1}$, a value of the same order of magnitude as that determined by the progress-curve analysis (Table 1).

The reversal of inhibition could also be demonstrated using a non-enzymic method. The complex was treated with α_1 -proteinase inhibitor, and samples from this mixture were withdrawn after 20, 90, 210, 300 and 380 min and electrophoresed in a polyacrylamide-gel gradient under non-denaturing conditions (Fig. 6). Free and elastase-bound α_1 -proteinase inhibitor and eglin c were visible on the gel. Fig. 6 shows the electrophoretic profile after 20 and 90 min of incubation. As can be seen, the band corresponding to the complex progressively faints while those representing free eglin c and elastase– α_1 -proteinase inhibitor complex progressively appear. These results thus provide

Scheme 3.

Fig. 7. Effect of increasing concentrations of eglin c on the elastolytic activity of $1 \mu\text{M}$ -elastase at pH 7.4 and 37°C

Order of addition of reagents: $\circ$, elastin+eglin c (incubated for 10 min)+elastase; $\bullet$, elastin+elastase (incubated for 3 min)+eglin c. Inset: kinetics of the solubilization of human aorta elastin (5 mg/ml) by $1 \mu\text{M}$ -elastase ($\triangle$) or $1 \mu\text{M}$ -elastase to which $0.5 \mu\text{M}$ -eglin c was added 3 min later ($\blacktriangle$). The concentration of soluble elastin peptides was measured by the absorbance at 280 nm of acidified and centrifuged portions of the reaction media [24]. The slopes of these curves were used to calculate the elastolytic activities.

Fig. 8. Effect of preincubation of elastase with elastin on the inhibition of elastolysis by eglin

Elastase ($1 \mu\text{M}$) was added to elastin (5 mg/ml) suspended in buffer A at 37°C . After the indicated preincubation times, $1 \mu\text{M}$ -eglin was added to the mixture and the rate of elastolysis was measured as indicated for Fig. 7 inset. The activity of an elastase control was used as reference (100% activity).

non-enzymic evidence for formation of a complex between elastase and eglin c and for reversibility of the binding.

Inhibition of the elastolytic activity of elastase by eglin c

The solubilization of human aorta elastin by elastase was linear with time as shown in the insert of Fig. 7. This allowed the calculation of rates of elastin solubilization in the absence and presence of eglin c. As can be seen in Fig. 7, the latter is a potent inhibitor of the elastolytic activity of elastase, which is fully abolished for an inhibitor/enzyme molar ratio of 1.00:1.25. Further, the effect was the same whether elastase was added to a suspension of eglin c+elastin or whether elastase was pre-incubated for 3 min with elastin before addition of eglin c.

Separate experiments, performed as described earlier for the elastin/neutrophil elastase system [14], showed that elastase was fully adsorbed on elastin in 2.5 min.

When elastase and elastin were preincubated for more than 3 min, eglin c was less efficient as an inhibitor. Fig. 8 shows the elastolytic activity of elastase preincubated for various periods before addition of an equimolar concentration of eglin c. As can be seen, the elastolytic activity increases with the preincubation time and becomes particularly important after 23 min of pre-incubation. Even higher eglin c concentrations did not achieve full inhibition.

DISCUSSION

The active-site loop of eglin c encompasses nine amino acid residues with P_1 being Leu-45 and P_2 being Asp-46 [25]. That P_1 is occupied by a leucine residue accounts for the poor binding of the inhibitor with trypsin-like enzymes and the strong binding with bovine pancreatic chymotrypsin ($K_i \sim 26 \text{ pM}$) [1]. Here we show that human pancreatic elastase, another chymotrypsin-like enzyme [17], also forms a tight complex with eglin c ($K_i = 0.37 \text{ nM}$). This result could not necessarily be predicted since chymase, the chymotrypsin-like proteinase from mast cells, has a rather high K_i for eglin c (44 nM) [26]. The progress-curve method used in the present paper was utilized previously by Baici & Seemüller [4] to monitor the inhibition of human leucocyte elastase by eglin c. The inhibition was found to be reversible and competitive, as shown here for human pancreatic elastase. Moreover, the rate and equilibrium constants characterizing the two eglin c-elastase complexes were of the same order of magnitude. Baici & Seemüller [4] also postulated the occurrence of a fast pre-equilibrium (Scheme 2), but, like us, they were unable to confirm it experimentally.

The second-order rate constant for the association of elastase with eglin c ($k_{\text{ass.}} = 7.3 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ at neutral pH) is two to three orders of magnitude lower than the maximum rate constant estimated for a bimolecular diffusion-controlled reaction [27]. In addition, the activation energy for the association process ($43.9 \text{ kJ} \cdot \text{mol}^{-1}$) is much higher than that for a diffusion step [28]. The interaction of elastase with eglin c is thus likely to occur via a fast pre-equilibrium complex followed by a slow first-order rearrangement of this complex (see Scheme 2). With the highest eglin c concentration used in this investigation ($1 \mu\text{M}$), the $k_{\text{ass.}}$ was $7.4 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Fig. 3), a value very close to those found with 10 nM and $0.4 \mu\text{M}$ inhibitor. This indicates that $K_i^* (\text{app.})$, the apparent dissociation constant of the postulated pre-equilibrium complex, is higher than $1 \mu\text{M}$. From the relationship $K_i^* (\text{app.}) = K_i^* (1 + [S_0]/K_m)$ it follows that K_i^* must be greater than $0.3 \mu\text{M}$. When such intermediate complexes could be detected for other enzyme/inhibitor systems, their dissociation constant was in the millimolar range (e.g. refs. [27] and [29]).

The hydrolysis of substrates by serine proteinases proceeds through the formation of an acyl-enzyme intermediate whose second-order rate of formation is given by $k_{\text{cat.}}/K_m$ (see ref. [30]):

Reversible protein proteinase inhibitors are thought to act in a substrate-like fashion, the stable EI complex being either the acyl-enzyme or the tetrahedral intermediate leading to its formation [31]. There is some parallelism between the pH-dependence of $k_{\text{ass.}}$ and that of $k_{\text{cat.}}/K_m$. However, the former could not be described by an acid-base titration curve. This might suggest that the association of elastase with eglin c is not controlled by the two ionizable groups of the enzyme that control the association of elastase with the substrate ($pK_1 = 5.9$; $pK_2 = 9.3$)

and/or that the EI complex is not an acyl-enzyme intermediate. The pH-dependency of k_{ass} may, however, also be controlled by ionizable groups that affect the conformation of eglin c. One of the three histidine residues of the inhibitor is exposed to solvent [25]. Its ionization between pH 5.0 and 8.0 may contribute to the observed pH-dependency of k_{ass} . Interestingly, however, X-ray crystallography shows that the P₁-P₂ peptide bond of eglin is not broken in the eglin c-subtilisin complex [25].

Reilly & Travis [32] have shown that pre-adsorption of neutrophil elastase to fibrous elastin renders the enzyme partially resistant to the inhibition by α_1 -proteinase inhibitor, a 52 kDa protein. We have made a similar observation with human pancreatic elastase [9]. This resistance is apparently related to the size of the inhibitor, since mucus proteinase inhibitor (11.7 kDa) readily inhibits elastin-bound human neutrophil [14] and pancreatic elastase [33]. On the other hand, eglin c (8.1 kDa) fully inhibits human neutrophil elastase pre-adsorbed on elastin [34]. Here we have extended these observations by demonstrating that eglin c strongly inhibits elastin-bound human pancreatic elastase. In addition, we have demonstrated that the inhibitor must be added a short time after the enzyme to get full inhibition of elastolytic activity. A prolonged contact of elastase with elastin significantly impairs the inhibitory potential of eglin c. Under these conditions, the latter is thus not more efficient than α_1 -proteinase inhibitor [9]. It is unclear why increasing amounts of the enzyme become resistant to inhibition as the elastolytic reaction proceeds. Perhaps during the initial stages of the reaction the enzyme molecules are bound on to the surface of the elastin fibres and are thus readily dissociated from this enzyme-substrate complex by the inhibitor. In contrast, once a significant number of peptide bonds are broken in elastin, part of the elastase molecules may be bound much tighter to other parts of the insoluble substrate and resist the dissociating action of the inhibitor. It must be emphasized that previous studies demonstrating the efficiency of small inhibitors on elastin-bound elastases were all performed using short times of elastin + elastase preincubation [14,33,34].

A characteristic feature of acute haemorrhagic pancreatitis is the destruction of elastic tissue in intra-pancreatic blood vessels with resultant bleeding. Human pancreatic elastase is thought to be responsible for these disorders [10]. Our kinetic data show that with a local eglin c concentration of approx. $7 \mu\text{M}$ free elastase would be inhibited in less than 1 s {the inhibition time, $d(t)$, is given by $d(t) = 5/k_{\text{ass}}[I_0]$; see ref. [35]}. This concentration is 2×10^4 -fold higher than K_i , so that eglin c will behave locally like a pseudo-irreversible inhibitor ($k_{\text{ass}}[I_0] \gg k_{\text{diss}}$). For these reasons, eglin c appears to be a very suitable elastase inhibitor for therapeutic purposes. Its allergenic potential (eglin c is a leech protein) may, however, limit its clinical use.

We thank CIBA-GEIGY (Basel, Switzerland) for the gift of recombinant eglin c and for partial financial support.

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