Kinetics of the inhibition of human leucocyte elastase by eglin from the leech *Hirudo medicinalis*

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The rate constants for the inhibition of human leucocyte elastase by eglin from the leech *Hirudo medicinalis* were determined by using a pre-steady-state kinetic approach. k_{on} and k_{off} for complex-formation and dissociation were $1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $8 \times 10^{-4} \text{ s}^{-1}$ respectively. K_i was calculated as the ratio $k_{off}/k_{on} = 8 \times 10^{-10} \text{ M}$, the binding of eglin to elastase was reversible and the inhibition mechanism was of the fully competitive type. The mechanistic properties of the system and the biological significance of the rate constants are discussed.

Eglin, an inhibitor miniprotein present in the leech *Hirudo medicinalis*, consists of a single polypeptide chain composed of 70 amino acid residues (Seemüller *et al.*, 1980). It is a potent inhibitor of the neutral proteinases elastase and cathepsin G from polymorphonuclear leucocytes (Seemüller *et al.*, 1981). These enzymes are thought to be involved in the pathogenesis of lung emphysema, clotting disorders and other inflammatory processes (Gadek *et al.*, 1980; Jochum *et al.*, 1983).

Under physiological conditions the proteolytic activity of leucocyte elastase is balanced by proteinase inhibitors present in plasma (α_1 -proteinase inhibitor) and other body secretions (anti-leucoproteinase). It has been suggested that, in certain inflammatory situations, the endogenous inhibitor capacity is insufficient, so that administration of suitable proteinase inhibitors might be a promising therapeutical approach (Jochum *et al.*, 1981).

Recently it has been shown that kinetic parameters may play an important role in evaluating the effectiveness of proteinase inhibitors *in vivo*. The significance of the kinetic constants for the interpretation of proteinase-inhibitor interactions under physiological and pathological conditions was discussed by Bieth (1980), who used half time and delay time of inhibition.

In this respect we were also interested in getting some idea of the effectiveness of eglin in inhibiting elastase. The kinetic constants k_{on} and k_{off} for

elastase-eglin complex-formation were determined by using a pre-steady-state kinetic analysis of reaction progress curves. The present paper reports that this method, previously employed for characterizing the interaction between cathepsin B and the low- M_r inhibitor leupeptin (Baici & Gyger-Marazzi, 1982), is also valid for the analysis of protein-protein interactions.

Materials and methods

Materials

Eglin C with a specific activity against chymotrypsin of 146 m-units (1 unit = $1 \mu\text{mol/min}$)/mg of protein (substrate succinyl-L-phenylalanine *p*-nitroanilide) was isolated from the leech *Hirudo medicinalis* as described previously (Seemüller *et al.*, 1977). Elastase (EC 3.4.21.37) was purified from human polymorphonuclear leucocytes as described elsewhere (Baici *et al.*, 1982) and the active-site concentration of this enzyme was determined with the azapeptide *N*-acetyl-L-alanyl-L-alanyl-azanorvaline *p*-nitrophenyl ester (Powers & Gupton, 1977). The elastase substrate Suc-Ala-Ala-Pro-Val-NMec was a gift of Dr. T. G. Payne, Sandoz Ltd., Basel, Switzerland.

Methods

Inhibition experiments with eglin were performed at a fixed concentration of elastase and variable concentrations of eglin and substrate. The progress of the reaction was monitored fluorimetrically by measuring the release of 7-amino-4-methylcoumarin from the fluorogenic

Abbreviation used: Suc-Ala-Ala-Pro-Val-NMec, succinyl-L-alanyl-L-alanyl-L-prolyl-L-valine 7-amino-4-methylcoumarylamide.

substrate Suc-Ala-Ala-Pro-Val-NMec. Fluorimetric measurements were performed with a recording Aminco SPF-500 fluorimeter operating in the ratio mode with techniques detailed previously (Baici & Gyger-Marazzi, 1982; Baici *et al.*, 1981), excitation and emission being at 383 and 455 nm respectively. The recorder scale was calibrated with 7-amino-4-methylcoumarin solutions of known concentration, the absorption coefficient of this substance at 342.5 nm being $9770 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Baici *et al.*, 1981).

Assays were performed in 50 mM-sodium/potassium phosphate buffer, pH 7.40, containing 0.05% (w/v) Triton X-100. By using elastase concentrations below 1 nM, a non-linear loss of activity was observed during the kinetic measurements and was likely due to adsorption of the enzyme on the surfaces of the cuvette. The adsorption was more pronounced in reaction mixtures containing elastase and substrate alone, whereas in the presence of eglin the reaction profiles remained linear for a very long time after attainment of the steady state. Artefacts of this type could be avoided by working in the presence of 0.05% (w/v) Triton X-100, by washing the cuvettes with an appropriate deter-

gent after each run, and by selecting observation times and enzyme concentrations so as to minimize these effects.

Results and discussion

Inhibition kinetics

Kinetic profiles for inhibition of human leucocyte elastase by eglin are shown in Fig. 1. Curve A shows the uninhibited reaction, whereas curves B and C represent the increase of product concentration with enzyme not preincubated or preincubated with the inhibitor respectively. The extrapolated values π , π^* , τ and τ^* are designated according to the nomenclature reported elsewhere (Baici & Gyger-Marazzi, 1982). The curves in Fig. 1 cannot be directly used for graphical calculations because the relative concentrations of enzyme and inhibitor violate both conditions $[E]_0 \ll [I]_0$ and $[I]_0 \gg K_i$ (Baici & Gyger-Marazzi, 1982). Fig. 1 is presented here to show the two fundamental experimental conditions (enzyme preincubated or not preincubated with the inhibitor) and clearly demonstrates that the reaction is reversible.

Both the lag phase, τ^* , and the intercept, π^* ,

Fig. 1. Inhibition kinetics of human leucocyte elastase by eglin

The curves represent continuous-recording traces for the release of 7-amino-4-methylcoumarin monitored fluorimetrically. A, control curve without inhibitor; B, reaction started by adding enzyme to a solution containing substrate and inhibitor; C, reaction started by adding substrate to a solution containing enzyme and inhibitor preincubated 15 min at 25°C. For all curves the substrate was Suc-Ala-Ala-Pro-Val-NMec at a final concentration of 0.54 mM in 50 mM-sodium/potassium phosphate buffer, pH 7.40, containing 0.05% (w/v) Triton X-100; the temperature was 25°C. The final concentration of the elastase active sites was 7.2 nM and that of eglin 18.3 nM. The extrapolated values of π , π^* and τ^* follow the nomenclature reported previously (Baici & Gyger-Marazzi, 1982). The extrapolation of the linear part of curve B on the time axis will give τ . These curves cannot be used directly for graphical calculations for the reasons explained in the text.

tend to zero with increasing inhibitor concentrations (Baici & Gyger-Marazzi, 1982). Moreover, the particular values of k_{on} and k_{off} , as it will be shown below, are such as to make experimental conditions practically impossible for the conditions $[E]_0 \ll [I]_0$ and $[I]_0 \gg K_i$ to be valid and, at the same time, give measurable values of π^* and τ^* . Preliminary estimations suggested K_i values in the range of 0.1–1 nM and, therefore, experiments suitable for graphical calculation were performed with inhibitor concentrations exceeding the upper value by at least a factor of 10 and with a fixed concentration of elastase of 0.9 nM. The curves in Fig. 1 could in principle be used with very complex iterative computer calculations taking into account the depletion of inhibitor due to complex-formation (Cha, 1980). This procedure is connected with several difficulties, which include the variation of five parameters in the iterative approximation, and was not considered by us. For the reasons listed above, equations containing the parameters π^* and τ^* could not be used. In contrast, the method of choice was the recording of the reaction profile in rate assays in which the enzyme was added to a mixture containing substrate and eglin (Fig. 2). These curves allowed the calculation of π by extrapolation of the linear part or by using the

method of Guggenheim (1926) applied to the initial phase of the reaction, where the rise in product concentration follows practically a pure exponential law (Baici & Gyger-Marazzi, 1982). The following equations were used for calculating preliminary values of the rate constants:

$$k_{on} = \frac{\pi(1 + \sigma)\lambda^2}{v_0[I]} \quad (1)$$

$$k_{off} = \frac{v_0 - v_i}{v_0} \cdot \frac{1}{|\tau|} \quad (2)$$

Eqn. (1) is derived from eqn. (6) in a previous paper (Baici & Gyger-Marazzi, 1982), to which the reader is referred for full details. λ is an apparent pseudo-first-order rate constant (s^{-1}) which describes the exponential approach to the steady state and is obtained as the slope of the Guggenheim plot. π is the amplitude of the exponential phase, σ the ratio $[S]/K_m$ and τ is the intercept of the linear part of the curve (steady state) with the time axis. v_0 and v_i are the steady-state reaction velocities in the absence and presence of inhibitor respectively.

The provisional estimates of k_{on} and k_{off} were then used as variable parameters in an iterative

Fig. 2. Inhibition of human leucocyte elastase by eglin

Continuous-recording traces monitored fluorimetrically as the release of 7-amino-4-methylcoumarin from the fluorogenic substrate Suc-Ala-Ala-Pro-Val-NMec. For all curves the substrate had a final concentration of 0.84 mM in 50 mM-sodium/potassium phosphate buffer, pH 7.40, containing 0.05% (w/v) Triton X-100; the temperature was 25°C. Since K_m for the substrate is 0.86 mM (Baici *et al.*, 1981), the ratio $\sigma = [S]/K_m$ used for calculations is 0.98. The reactions were all started by adding enzyme to a final concentration of active sites of 0.9 nM. The final concentrations of eglin (in nM) were 0 (A), 13.7 (B), 22.9 (C), 34.4 (D) and 45.8 (E).

best-fit calculation to the following equation (Baici & Gyger-Marazzi, 1982):

$$P = \frac{v_0 k_{\text{off}}}{\lambda} t + \frac{v_0 k_{\text{on}} [I]}{(1 + \sigma) \lambda^2} (1 - e^{-\lambda t}) \quad (3)$$

in which [P] represents the concentration of the reaction product. During computation v_0 , σ and [I] were taken as known parameters. The calculated curves were drawn on transparent graph paper, superimposed to the experimental curves and k_{off} and k_{on} varied until the calculated and experimental curves reached a maximum of overlapping.

By using this method of analysis, experiments such as those shown in Fig. 2 and run in triplicate at several inhibitor concentrations afforded the following values ($\pm$ s.d.) for the rate constants: $k_{\text{on}} = 1.0 (\pm 0.4) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $k_{\text{off}} = 8.0 (\pm 1.6) \times 10^{-4} \text{ s}^{-1}$. From the rate constants, K_i can be calculated as the ratio $k_{\text{off}}/k_{\text{on}} = 8 \times 10^{-10} \text{ M}$. The reversibility of the reaction, a necessary prerequisite for the applicability of eqns. (1) and (2), was demonstrated by the property of the EI complex to be dissociated in the presence of substrate (Fig. 1).

Mechanistic considerations

The results demonstrate that the inhibition mechanism of elastase by eglin is fully competitive and reversible (Scheme 1). Because of the particular values of k_{on} and K_i , true steady-state kinetic studies are virtually impossible. The competitive character of the inhibition was unequivocally demonstrated by performing experiments at various substrate concentrations (results not shown); it was found that, at fixed eglin concentrations, the

Scheme 1

Scheme 2

degree of inhibition decreased with increasing substrate concentrations and the inhibition was complete in the presence of excess eglin. These are peculiar characteristics of the fully competitive mechanism. For qualitative purposes only, the specific-velocity plot (Baici, 1981) was applied to the slopes of inhibited reaction profiles obtained with elastase preincubated (or not) with eglin under the condition $[E]_0 \ll [I]_0$, which implies also $[I]_0 \cong [I]$. The extrapolation procedure suggested K_i to be in the range 0.5–1.5 nM, in agreement with the value of 0.8 nM obtained by pre-steady-state kinetics.

Experimental conditions suitable for analysis with the Dixon plot for tightly bound inhibitors (Dixon, 1972) are difficult to meet in the elastase–eglin system. Since one is compelled to work at relatively high enzyme concentrations, the curve obtained is essentially a titration profile, from which an estimation of K_i can be obtained by the method of Green & Work (Seemüller *et al.*, 1977; Green & Work, 1953).

We are aware of the fact that Scheme 1 is an oversimplification of the inhibition mechanism. In fact, the reaction might proceed by the formation of several intermediates, each of them being characterized by a set of microscopic constants. An overall mechanism of the type shown in Scheme 2 has been reviewed by Laskowski & Kato (1980) and is in principle a likely one for elastase and eglin. I and I^* would be the virgin and modified inhibitors respectively, and L and L^* loose non-covalent complexes, X a relatively long-lived intermediate, and C a stable enzyme–inhibitor complex. The direct kinetic detection of L, C and X remains obscure and is not accessible with our analytical method. The pre-steady-state analysis presented here can probably tell us what happens up to step C (or X?) and the determined k_{on} is in this case an average rate constant composed of all the microscopic constants of the various steps, as indicated in Scheme 1. In this Scheme we also suggest a transformation, analogous to that in Scheme 2, of the inhibitor after complex-formation. The transformation is difficult to demonstrate kinetically, since very long incubation times are necessary. During this time other effects may interfere with the assays, thus producing artefacts. A possible source of error is the de-activation of enzyme at the very low concentrations employed (denaturation, adsorption, etc.), so that the preincubated mixture may contain less activity than expected. On the other hand, the inhibitory action of I^* could either be better or worse with respect to I. The degree of dissociation of I^* from the enzyme is unknown, but one should expect a regain of enzyme activity if the dissociation really takes place. The net effect will be an average of the single contributions.

Some of the experiments performed during the present study suggested a mechanism compatible with the loop shown at the bottom of Scheme 1, but in a purely qualitative way. Another piece of evidence comes from observations made during electrophoresis of the elastase–eglin complex. The enzyme and inhibitor were preincubated and then subjected to electrophoresis in the presence and absence of sodium dodecyl sulphate as described by Seemüller *et al.* (1977) for the chymotrypsin–eglin system. Only in the presence of the denaturing agent could the dissociation of modified inhibitor be observed. This experiment supports the hypothesis that the EI* complex is a likely one and that I* can be dissociated either in the presence of excess substrate or after denaturation of the complex.

Biological significance of the rate constants

The association rate constant, k_{on} , permits calculation of the time required for inhibiting the enzyme. The importance of the delay time of inhibition has been stressed by Bieth (1980). The half-time for inhibition will have different values depending on the concentration of the inhibitor. With I in great excess of the enzyme the reaction will have a pseudo-first-order character and $t_{\frac{1}{2}}$ will be $0.69/(k_{on}[I])$. With I concentrations not much exceeding the concentration of enzyme, the reaction will have a second-order character and $t_{\frac{1}{2}}$, in the limiting case of $[I]_0 = [E]_0$, will be $1/(k_{on}[I])$. Therefore, for qualitative purposes, we can assume $t_{\frac{1}{2}} \cong 1/(k_{on}[I])$ for an estimation of the delay times for inhibiting elastase with eglin. It should be noted that this delay time is actually the time required to form the EI complex. Once EI is formed, since the inhibition mechanism is fully competitive, the degree of inhibition will depend on both the concentrations of inhibitor and substrate. In any case, for $[I]/K_i > 10$ at least and $[S]/K_m \leq 1$, the inhibition will be practically complete and the delay times for complex-formation will coincide with the delay times for inhibition. Table 1 shows the times required for achieving a given percentage of inhibition for various eglin concentrations. The Table clearly demonstrates that at concentrations between $1 \mu\text{M}$ and $10 \mu\text{M}$ the inhibition will be significantly fast. For instance, with eglin $2 \mu\text{M}$, the inhibition will be 75% after 1 s and 94% after 2 s.

Table 1. Delay times for inhibition of human leucocyte elastase by eglin

The numbers in the Table represent the time in seconds required for obtaining the percentages of inhibition (% inh.) indicated, for various eglin concentrations. Calculated on the basis of $k_{on} = 1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$.

[I] (μM)	Time (s)		
	After $1 \times t_{\frac{1}{2}}$ (50.0% inh.)	After $3 \times t_{\frac{1}{2}}$ (87.5% inh.)	After $5 \times t_{\frac{1}{2}}$ (96.8% inh.)
0.1	10	30	50
1.0	1	3	5
2.0	0.5	1.5	2.5
10.0	0.1	0.3	0.5

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