Kinetics of the interaction of chymotrypsin with eglin c

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The kinetics of binding of recombinant eglin c to bovine pancreatic chymotrypsin was studied by conventional and stopped-flow techniques. With nanomolar enzyme and inhibitor concentrations, the inhibition was fast and pseudo-irreversible ($k_{\text{assoc.}} = 4 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 7.4 and 25 °C). Reaction of the enzyme-inhibitor complex with α_1 -proteinase inhibitor, an irreversible chymotrypsin ligand, resulted in a slow release of free eglin c, which was monitored by electrophoresis ($k_{\text{dissoc.}} \approx 1.6 \times 10^{-8} \text{ s}^{-1}$, $t_{1/2} \approx 5$ days). The proflavin displacement method and a stopped-flow apparatus were used to monitor the association of chymotrypsin with eglin c under a wide range of inhibitor concentration and under pseudo-first-order conditions. At pH 7.4 and 25 °C or 5 °C, or at pH 5.0 and 25 °C, the pseudo-first-order rate constant of proflavin displacement increased linearly with eglin c up to the highest concentration tested, suggesting a one-step bimolecular association reaction: $\text{E} + \text{I} \rightleftharpoons \text{EI}$. However, $k_{\text{assoc.}}$ is much lower than the rate constant for a bimolecular reaction and its activation energy ($66 \text{ kJ} \cdot \text{mol}^{-1}$ at pH 7.4 and $78 \text{ kJ} \cdot \text{mol}^{-1}$ at pH 5.0) is far too high for a diffusion-controlled step. The enzyme-inhibitor association may therefore occur via a loose pre-equilibrium complex EI^* ($K_1^* \gg 5 \times 10^{-4} \text{ M}$) that rapidly isomerizes ($k_2 \gg 2 \times 10^3 \text{ s}^{-1}$) into an extremely stable final complex ($K_1 \approx 4 \times 10^{-13} \text{ M}$). Unlike other proteinase-inhibitor systems, the chymotrypsin-eglin association is virtually pH-independent.

INTRODUCTION

Eglin c, an 8.1 kDa protein, is a serine-proteinase inhibitor first isolated from the leech *Hirudo medicinalis* [1] and now produced by genetic engineering [2] for therapeutic purposes. Recombinant eglin c is indistinguishable from the natural molecule with respect to its immunological and kinetic properties [3], although its *N*-terminal amino acid residue is acetylated. It tightly inhibits neutrophil elastase, neutrophil cathepsin G, pancreatic chymotrypsin and subtilisin, but weakly interacts with trypsin or porcine pancreatic elastase and is inactive on plasmin, thrombin and kallikrein [1]. The kinetic analysis of its interaction with neutrophil elastase indicates that the equilibrium dissociation constant of the complex is $2.7 \times 10^{-12} \text{ M}$ [4]. Owing to this high inhibitory potency, eglin c was thought to be a potential drug against inflammation, cystic fibrosis or emphysema, diseases in which neutrophil elastase is involved [5].

Eglin c shares sequence identities with two chymotrypsin inhibitors from barley (*Hordeum vulgare*) grains [6], with potato (*Solanum tuberosum*) inhibitor I [7] and with two proteinase B inhibitors from yeast (*Saccharomyces cerevisiae*) [8]. Partial amino-acid-sequence identities established several families of serine-proteinase inhibitors [9]. Eglin c belongs to the potato inhibitor I family characterized by a small number of disulphide bridges. Actually, eglin c has no disulphide bridges at all, and yet it is a remarkably stable protein [3].

Eglin c in complex with subtilisin Carlsberg is a wedge-shaped disc with the active-site loop at the narrow end of the wedge [10]. This loop encompasses nine amino acid residues with $\text{P}_1 = \text{Leu-45}$ and $\text{P}'_1 = \text{Asp-46}$ and its conformation is stabilized by interactions with arginine side chains [11]. The nature of the P_1 residue explains why the inhibitor poorly binds trypsin and strongly interacts with chymotrypsin. The inhibition of the latter by eglin c has been studied by Seemüller *et al.* [1], who used two substrates with different susceptibilities to chymotrypsin. Both titration curves were linear, indicating good affinity. A precise value for the equilibrium dissociation constant of the complex

was, however, not given. Also, association and dissociation rate constants were not measured.

Here we describe the measurement of these parameters as well as stopped-flow experiments aimed at testing whether the stable chymotrypsin-eglin complex is preceded by a loose pre-equilibrium complex as is the case with members of the soybean-trypsin-inhibitor and the pancreatic-trypsin-inhibitor families of proteinase inhibitors [13,14].

EXPERIMENTAL

Materials

Three-times-crystallized bovine α -chymotrypsin was obtained from Worthington Biochemical Corp, Freehold, NJ, U.S.A., and was active-site-titrated with *N*-trans-cinnamoylimidazole (Sigma, St. Louis, MO, U.S.A.) as described by Schonbaum *et al.* [14]. Proflavin was purchased from Mann Research Laboratories, New York, NY, U.S.A. Recombinant eglin c was generously given by Dr. H. P. Schnebli (Ciba-Geigy, Basel, Switzerland) and was active-site-titrated with active-site-titrated chymotrypsin and leucocyte elastase as described previously [4]. Succinyl-Ala₂-Pro-Phe *p*-nitroanilide (Suc-Ala₂-Pro-Phe-*p*NA) [15] and Suc-Ala₂-Phe-*p*NA [16], two chymotrypsin substrates, were purchased from Bachem, Bubendorf, Switzerland. Human plasma α_1 -proteinase inhibitor ($\alpha_1\text{PI}$) was isolated and active-site-titrated as described previously [17]. Throughout the present paper all concentrations of enzyme and inhibitor are given in molarities of active proteins. Most of the kinetic experiments were done in 50 mM-Hepes/100 mM-NaCl, pH 7.4, at 25 °C.

Methods

Kinetic measurements using proflavin. Most rate constants for the association of chymotrypsin with eglin c were measured using the proflavin-displacement method [13,18,19] and a stopped-flow apparatus (Hi-Tech Scientific SF/PQ 53, Salisbury, Wilts., U.K.) on line with a Hewlett-Packard model 9000 microcomputer. The path length of the observation cell was 10 mm, and the dead time

Abbreviations used: Suc, succinyl; *p*NA, *p*-nitroanilide; $\alpha_1\text{PI}$, α_1 -proteinase inhibitor.

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of the apparatus was about 1 ms. One syringe contained 2 μM -enzyme and the other was filled with inhibitor (20–2000 μM) and 60 μM -proflavin.

The equilibrium dissociation constant (K_p) of the proflavin–chymotrypsin complex was measured by differential spectrophotometry at 465 nm [19,20] using a Cary 2200 spectrophotometer. The measurements were made with constant concentrations of proflavin (2 μM) and increasing concentrations of chymotrypsin (20–160 μM). Differential spectrophotometry at 465 nm also showed that eglin c displaced proflavin stoichiometrically from its complex with chymotrypsin, indicating that the dye and the inhibitor are competing ligands for the enzyme. Control experiments showed that free or chymotrypsin-bound eglin c did not bind proflavin. The decrease of absorbance at 465 nm could thus be used to monitor the association of eglin c with chymotrypsin.

Kinetic measurements using substrates. Synthetic substrates were also used to assess the kinetics of binding of eglin c to chymotrypsin. In one set of experiments, equimolar concentrations of enzyme and inhibitor (5 nM) were allowed to react for given periods of time before addition of 10 μl of 30 mM-Suc-Ala₂-Pro-Phe-pNA (dissolved in *NN*-dimethylformamide) to 990 μl of reaction mixture. In these experiments the substrate measures the residual enzyme activity and stops the association process, since its concentration is 6-fold higher than K_m [15]. The residual enzyme activities were measured at 410 nm and 25 °C and were used to calculate the concentrations of unchanged enzyme at the time of addition of substrate.

In another set of experiments, the binding of chymotrypsin to eglin c was allowed to proceed in the presence of the less sensitive and more soluble substrate Suc-Ala₂-Phe-pNA. The enzyme (0.7 nM) was added to a mixture of 7 nM-inhibitor and 7 mM-substrate and the release of product (A_{410}) was recorded with a Cary 2200 spectrophotometer on line with an IBM PS/2 model 30 microcomputer.

Dissociation of the chymotrypsin–eglin c complex with $\alpha_1\text{PI}$. This experiment was done in a buffer containing 50 mM-Hepes, 100 mM-NaCl and 20 μM -NaN₃ at pH 7.4 and at 25 °C. The complex (20 μM) was allowed to react with 30 μM - $\alpha_1\text{PI}$, and aliquots of this mixture were withdrawn at selected times and electrophoresed in a 8–25%-polyacrylamide gradient gel from Pharmacia (Les Ulis, France). Automatic electrophoresis and Coomassie Blue staining were done with the Phastsystem apparatus from Pharmacia within 1 h. A control sample of 20 μM -eglin c was electrophoresed on each gel to serve as an indicator for 100% complex dissociation. The intensities of the protein bands were quantified by densitometry scanning using a Shimadzu CS 930 scanner.

RESULTS

Eglin c as a tight-binding pseudo-irreversible inhibitor of chymotrypsin

The enzyme–inhibitor interaction was first studied using low reactant concentrations in order to measure the overall classical rate constants $k_{\text{assoc.}}$ and $k_{\text{dissoc.}}$ shown in Scheme 1:

Scheme 1

In one set of kinetic experiments the inhibition of chymotrypsin by eglin c was studied under second-order conditions ($[\text{E}_0] = [\text{I}_0] = 5 \text{ nM}$) in the absence of substrate as described in the legend to Fig. 1. The inhibition was virtually complete in 10³ s, and the

data could be fitted to the following second-order irreversible binding equation (data not shown):

$$[\text{E}] = \frac{[\text{E}_0]}{1 + [\text{E}_0] \cdot k_{\text{assoc.}} \cdot t} \quad (1)$$

To better illustrate the irreversible nature of the binding, we plotted the data according to the double-reciprocal form of eqn. (1):

$$\frac{1}{[\text{E}]} = \frac{1}{[\text{E}_0]} + k_{\text{assoc.}} \cdot t \quad (2)$$

As Fig. 1 shows, this plot is linear for 17 half-lives, indicating that complex dissociation, if any, does not take place during the duration of this experiment. The $k_{\text{assoc.}}$ calculated from this experiment was $4.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$.

In order to detect complex dissociation, we measured the kinetics of enzyme–inhibitor binding in the presence of a substrate. Suc-Ala₂-Phe-pNA was used for this purpose because it is more water-soluble and less sensitive than Suc-Ala₂-Pro-Phe-pNA [16]. Hence a very large excess of substrate could be used ($[\text{S}_0] = 67 K_m$) and substrate depletion was negligible during the long duration of the experiment (6 h). Enzyme (E) was added to a mixture of inhibitor (I) and substrate (S) and the release of

Fig. 1. Kinetics of inhibition of chymotrypsin by eglin c studied under second-order conditions in the absence of substrate at pH 7.4 and 25 °C

Equimolar amounts of enzyme and inhibitor (5 nM) were mixed and Suc-Ala₂-Pro-Phe-pNA (0.3 mM, 6 K_m) was added after various periods of time to stop the association process and to measure the residual enzyme activity, which was used to calculate the concentration of free enzyme. The data were plotted in accordance with eqn. (2). $t_{1/2}$ was 45 s.

Fig. 2. Progress curve for the inhibition of chymotrypsin by eglin c under pseudo-first-order conditions at pH 7.4 and 25 °C

The enzyme was added to a mixture of inhibitor and substrate and the release of *p*-nitroaniline (ϵ 8800 $\text{M}^{-1} \cdot \text{cm}^{-1}$) was recorded at 410 nm. Final concentrations were as follows: [chymotrypsin] = 0.7 nM, [eglin c] = 7 nM, [Suc-Ala₂-Phe-pNA] = 7 mM (67 K_m).

product (P) was recorded. Analysis of the progress curve was based on a reaction scheme previously used for the eglin c-pancreatic elastase interaction [21]:

Scheme 2

A typical curve is shown in Fig. 2. Since $[I_0] \gg [E_0]$ and $[P] \ll [S_0]$, the progress curve could be described by the following equation [22]:

$$P = v_s t + (v_0 - v_s)(1 - e^{-kt})/k \quad (3)$$

where P is the product concentration, v_0 the velocity at $t = 0$, v_s the steady-state velocity and k the apparent first-order rate constant for the approach to the steady state. We used low enzyme and inhibitor concentrations (0.7 nM and 7 nM respectively) together with a high substrate concentration ($67 K_m$) to favour the dissociation of EI. Progress curves such as that shown in Fig. 2 were fitted to eqn. (3) by non-linear regression analysis to obtain the best estimates of v_0 ($1.48 \times 10^{-8} \text{ M} \cdot \text{s}^{-1}$), v_s ($1.28 \times 10^{-10} \text{ M} \cdot \text{s}^{-1}$) and k ($4.15 \times 10^{-4} \text{ s}^{-1}$). The following relationships were used to calculate $k_{\text{assoc.}}$ and $k_{\text{dissoc.}}$ from v_0 , v_s and k [23]:

$$k = k_{\text{assoc.}}[I_0]/(1 + [S_0]/K_m) + k_{\text{dissoc.}} \quad (4)$$

$$k_{\text{dissoc.}} = k \cdot (v_s/v_0) \quad (5)$$

The following constants were found: $k_{\text{assoc.}} = 4.0 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$; $k_{\text{dissoc.}} \approx 3.6 \times 10^{-6} \text{ s}^{-1}$. The former is in excellent agreement with the value measured above under second-order conditions. The latter must be considered as tentative, because its magnitude is only about 1% that of k .

To demonstrate clearly the reversibility of the binding and to better estimate $k_{\text{dissoc.}}$ we used $\alpha_1\text{PI}$ to dissociate the EI complex. The dissociation reaction is summarized in Scheme 3:

Scheme 3

$\alpha_1\text{PI}$ is an irreversible and fast-acting inhibitor of chymotrypsin ($k'_{\text{assoc.}} = 5.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$) [24] which should effectively shift the $\text{E} + \text{I} \rightleftharpoons \text{EI}$ equilibrium. The EI complex was allowed to react with $\alpha_1\text{PI}$, and samples from the mixtures were withdrawn and electrophoresed. Fig. 3(a) shows typical gels run at zero time and after 65 and 136 h of incubation. As can be seen, the bands corresponding to free eglin and to the $\alpha_1\text{PI}$ -chymotrypsin complex progressively increase in intensity. Densitometry scanning allowed us to estimate the concentration of free inhibitor, which is plotted as a function of time in Fig. 3(b). The dissociation data could not be fitted to a simple exponential as would be expected for a first-order dissociation process. This may be due to: (i) the low degree of dissociation; only about 40% eglin c was freed from the complex at the end of the dissociation reaction

(5.5 days); (ii) the semi-quantitative technique used to assay free eglin c; and (iii) the concentration of the dissociation agent; although $k'_{\text{assoc.}}$ is 50% larger than $k_{\text{assoc.}}$ (Scheme 3) and $[\alpha_1\text{PI}]$ was 50% greater than $[\text{EI}]$ in the dissociation experiment, competition between free I and $\alpha_1\text{PI}$ for the binding of E must have taken place after some eglin c was released. Hence the relationship $d[\text{I}]/dt = k_{\text{dissoc.}}[\text{EI}]$ (first-order release of inhibitor) is only valid during the very beginning of the dissociation reaction. Higher $\alpha_1\text{PI}$ concentrations gave electrophoretic problems. We therefore used the initial-rate method to derive $k_{\text{dissoc.}}$: initial rate of inhibitor release $= k_{\text{dissoc.}}[\text{EI}_0]$. The initial rate, as estimated from the slope of the broken line of Fig. 3(b), was $\approx 3.2 \times 10^{-11} \text{ M} \cdot \text{s}^{-1}$. From this a tentative value of $1.6 \times 10^{-6} \text{ s}^{-1}$ was found for $k_{\text{dissoc.}}$ ($t_{1/2} \approx 5$ days). This value is in fair agreement with the tentative value obtained with the progress-curve method. The K_i calculated from $k_{\text{dissoc.}}/k_{\text{assoc.}}$ was about $4.0 \times 10^{-13} \text{ M}$. Eglin c is thus a very tight-binding reversible chymotrypsin inhibitor which exhibits pseudo-irreversible behaviour at low enzyme and inhibitor concentrations.

Effect of eglin c concentration on the rate constant for the chymotrypsin-eglin c association. The proflavin-displacement method and a stopped-flow apparatus were used to measure the rate of association of chymotrypsin with eglin c with a wide range of inhibitor concentrations in order to decide whether the two partners associate according to a simple bimolecular reaction (Scheme 4a) or via an intermediate (Scheme 4b). The binding may be considered as irreversible for reasons outlined above.

Scheme 4a

Scheme 4b

The rate of displacement of proflavin (Pf) from its complex with chymotrypsin was measured under the following conditions: $[I_0] \gg [E_0]$, $[E_0] \ll K_p$, the equilibrium dissociation constant of the chymotrypsin-proflavin complex and $[\text{Pf}_0] \approx K_p$. Pf_0 is the total proflavin concentration. Under such conditions, the decrease in absorbance at 465 nm reliably measures the binding of E to I and the association kinetics are pseudo-first-order [13]. The typical stopped-flow trace shown in Fig. 4 demonstrates that the decrease in absorbance at 465 nm is indeed first-order. The apparent pseudo-first-order rate constant, $k_{\text{obs.}}$, derived from such a trace is given by:

$$k_{\text{obs.}} = \frac{k_{\text{assoc.}}[I_0]}{1 + [\text{Pf}_0]/K_p} \quad (\text{Scheme 4a}) \quad (6)$$

$$k_{\text{obs.}} = \frac{k_2[I_0]}{[I_0] + K_1^*(\text{app.})} \quad (\text{Scheme 4b}) \quad (7)$$

where $K_1^*(\text{app.}) = k_{-1}/k_1 (1 + [\text{Pf}_0]/K_p)$. Eqn. (7) predicts a linear dependence of $k_{\text{obs.}}$ on $[I_0]$, whereas according to eqn. (8), this dependency is hyperbolic.

The $k_{\text{obs.}}$ -versus- $[I_0]$ relationship was first investigated at pH 7.4 and 25 °C, conditions under which $k_{\text{obs.}}$ increased linearly with $[I_0]$ (results not shown). The highest value of $k_{\text{obs.}}$ (230 s^{-1}) was

Fig. 3. Dissociation of the chymotrypsin–eglin c complex (20 μM) by $\alpha_1\text{PI}$ (30 μM) at pH 7.4 and 25 $^{\circ}\text{C}$

Aliquots were removed, electrophoresed, and stained after selected periods of time. The amounts of free eglin c were quantified by densitometry scanning. (a) Typical electrophoretic patterns. Lanes 1–3, 0, 65 and 136 h of incubation respectively; lane 4, eglin c control (CHY, chymotrypsin). (b) Plot of free eglin c/total eglin c as a function of time. The slope of the tangent to the curve (broken line) was used to calculate the initial rate of eglin c appearance.

obtained with $[I_0] = 100 \mu\text{M}$ and corresponds to $t_{1/2}$ of about 3 ms, a value close to the dead time of the apparatus. In order to test higher eglin c concentrations, we used experimental conditions under which the rate of association was supposedly lower. At pH 7.4 and 5 $^{\circ}\text{C}$, k_{obs} again increased linearly with $[I_0]$ up to the highest concentration tested (1 mM; see Fig. 5). The same was true at pH 5.0 (50 mM-acetate/100 mM-NaCl) and 25 $^{\circ}\text{C}$.

Thus the eglin c–chymotrypsin association does apparently not involve a reaction intermediate. The value of k_{assoc} , calculated from the slope of the k_{obs} -versus- $[I_0]$ plot was $4.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 7.4 and 25 $^{\circ}\text{C}$. This value is in excellent agreement with those measured at low reactant concentrations, namely 4.0×10^6 and $4.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$.

Effect of temperature and pH on k_{assoc} . This was measured with the proflavin-displacement method. To derive k_{assoc} from k_{obs} (eqn. 7), we determined a number of K_p values (Table 1) and extrapolated the others.

The effect of temperature was studied at pH 5.0 and 7.4. The Arrhenius plots (Fig. 6) yielded two straight lines from which activation energies of 78 $\text{kJ} \cdot \text{mol}^{-1}$ (pH 5.0) and 66 $\text{kJ} \cdot \text{mol}^{-1}$ (pH 7.4) were calculated.

The k_{assoc} reaches a maximum of $4.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ near neutral

Fig. 4. Kinetics of displacement of proflavin from the chymotrypsin–proflavin complex by eglin c as monitored at 465 nm (pH 7.4, 25 $^{\circ}\text{C}$)

The final concentrations of the reagents were: [chymotrypsin] = 1 μM ; [eglin c] = 10 μM , [proflavin] = 30 μM . The solid line represents the best exponential fit to the curve ($k_{\text{obs}} = 21.5 \text{ s}^{-1}$).

Fig. 5. Influence of inhibitor concentration on the apparent first-order rate constant (k_{obs}) for the binding of chymotrypsin to eglin c at pH 7.4 and 5 $^{\circ}\text{C}$

k_{obs} was measured as described in Fig. 4. The concentrations of enzyme and proflavin used throughout were 1 μM and 30 μM respectively.

pH (Fig. 7). Below and above this pH optimum, k_{assoc} weakly decreases up to $2.4 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 5.0 and $2.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 9.0. The bell-shaped profile of the k_{assoc} -versus-pH curve suggests that at least two ionization groups are involved in the enzyme–inhibitor interaction. The data could, however, not be interpreted in terms of simple acid–base ionizations. To rationalize the very small variation of k_{assoc} with pH, we hypothesized that the binding capacity of one reaction partner does not change with pH, whereas the other retains about 45% of its binding ability either if one of its acidic group is fully

Table 1. Dissociation constants (K_p) of the chymotrypsin–proflavin complex at various temperature and pH values

I was 0.1 throughout.

Temperature ($^{\circ}\text{C}$)	pH...	K_p (μM)					
		5	6	7	7.4	8	9
5		34.1 $\pm$ 6.1			16.0 $\pm$ 1.0		
25		89.1 $\pm$ 6.1	55.6 $\pm$ 5.8	34.2 $\pm$ 3.3	28.1 $\pm$ 2.3	41.2 $\pm$ 4.3	60.1 $\pm$ 2.9
37		180 $\pm$ 18			38.7 $\pm$ 2.7

Fig. 6. Arrhenius plots for the influence of temperature on $k_{\text{assoc.}}$ at two different pH values

The $k_{\text{assoc.}}$ was measured by using the proflavin-displacement method. Activation energies were $66 \text{ kJ} \cdot \text{mol}^{-1}$ and $77 \text{ kJ} \cdot \text{mol}^{-1}$ at pH 7.4 and 5.0 respectively. $\circ$, pH 7.4; $\bullet$, pH 5.0.

Fig. 7. pH-dependence of the association rate constant $k_{\text{assoc.}}$ at 25°C as measured by the proflavin-displacement method

The following buffers were used: 50 mM-acetate/100 mM-NaCl, pH 5.0 and 5.5; 50 mM-Mes/100 mM-NaCl, pH 6.0 and 6.5; 50 mM-Hepes/100 mM-NaCl, pH 7.0 and 7.4; 50 mM-Tris/100 mM-NaCl, pH 8.0–9.0. The solid line was calculated with eqn. (9), whose parameters were determined by non-linear regression analysis.

protonated or if one of its basic group is fully deprotonated. The following equation takes into account these assumptions:

$$k_{\text{assoc.}} = \frac{k_{\text{assoc. (0)}}}{(1 + 10^{pK_1 - \text{pH}})(1 + 10^{\text{pH} - pK_2})} + \frac{k_{\text{assoc. (a)}}}{(1 + 10^{\text{pH} - pK_1})(1 + 10^{\text{pH} - pK_2})} + \frac{k_{\text{assoc. (b)}}}{(1 + 10^{pK_1 - \text{pH}})(1 + 10^{pK_2 - \text{pH}})} \quad (8)$$

where $k_{\text{assoc. (0)}}$ is the maximal association rate constant (i.e. $k_{\text{assoc.}} = k_{\text{assoc. (0)}}$ if $\text{pH} = (pK_1 + pK_2)/2$), $k_{\text{assoc. (a)}}$ is the minimal rate constant if $\text{pH} < pK_1$, and $k_{\text{assoc. (b)}}$ is the minimal rate constant when $\text{pH} > pK_2$. The best estimates of pK_1 , pK_2 , $k_{\text{assoc. (0)}}$, $k_{\text{assoc. (a)}}$ and $k_{\text{assoc. (b)}}$ were determined by a non-linear regression fit to eqn. (8). The theoretical curve shown in Fig. 7 corresponds to $pK_1 = 6.3$, $pK_2 = 8.1$, $k_{\text{assoc. (0)}} = 4.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{\text{assoc. (a)}} = 2.4 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{\text{assoc. (b)}} = 2.05 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$.

DISCUSSION

The current data show that eglin c is a fast-acting and tight-binding inhibitor of bovine pancreatic chymotrypsin. The second-order association rate constant, $k_{\text{assoc.}}$, was determined by using three different techniques and widely different enzyme concen-

trations: (i) a pseudo-first-order method in the presence of a substrate with $[E_0] = 0.7 \text{ nM}$; (ii) a second-order method with no substrate present during the association process and with $[E_0] = 5 \text{ nM}$; (iii) a pseudo-first-order method in the presence of proflavin and with $[E_0] = 1 \text{ } \mu\text{M}$. These techniques yielded very close $k_{\text{assoc.}}$ values $[(4.0\text{--}4.3) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}]$. Reversal of inhibition could be achieved by allowing the enzyme–inhibitor complex to react with $\alpha_1\text{PI}$, a fast-acting irreversible chymotrypsin ligand [24]. The slow appearance of free eglin c, as monitored by electrophoresis, allowed a rough estimate of $k_{\text{dissoc.}}$ to be made ($1.6 \times 10^{-6} \text{ s}^{-1}$, $t_{1/2} \approx 5$ days). The eglin c–chymotrypsin complex is thus extremely stable. $K_i \approx 4.0 \times 10^{-13} \text{ M}$, $\Delta G_0 = -70 \text{ kJ} \cdot \text{mol}^{-1}$. This tight binding explains why reaction of constant amounts of chymotrypsin with increasing amounts of eglin c yielded straight inhibition curves [1].

The active-site loop of eglin c encompasses nine amino acid residues, with P_1 being Leu-45 and P'_1 being Asp-46 [11]. That P_1 is occupied by a leucine residue accounts for the poor binding of eglin c with trypsin and trypsin-like enzymes from the coagulation and kinin systems [1] and the very strong binding of bovine pancreatic chymotrypsin, an enzyme that rapidly hydrolyses substrates with $P_1 = \text{Leu}$. Human pancreatic elastase, another member of the chymotrypsin family, binds less tightly to eglin c than chymotrypsin ($k_{\text{assoc.}} = 7.3 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{\text{dissoc.}} = 2.7 \times 10^{-4} \text{ s}^{-1}$, $K_i = 3.7 \times 10^{-10} \text{ M}$) [21]. Moreover, chymase, the chymotrypsin-like proteinase from mast cells, forms a rather loose complex with the inhibitor ($K_i = 4.4 \times 10^{-8} \text{ M}$). On the other hand, human neutrophil elastase, which slowly hydrolyses substrates with $P_1 = \text{Leu}$ [25,26], forms a very tight complex with eglin c ($k_{\text{assoc.}} = 1.3 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{\text{dissoc.}} = 3.5 \times 10^{-5} \text{ s}^{-1}$, $K_i = 2.7 \times 10^{-12} \text{ M}$) [4]. It is therefore likely that the P_1 – S_1 complementarity does not significantly contribute to the overall proteinase–eglin c binding energy, but may solely serve for the initial enzyme–inhibitor recognition. Most of the high binding energy of the complexes of eglin with chymotrypsin or elastase probably arises from P–S interactions different from P_1 – S_1 .

Laskowski and co-workers proposed a standard mechanism for the interaction of proteinases with reversible proteinase inhibitors [9]:

Scheme 5

where I^* is a proteolytically modified form of I , L and L^* are loose and rapidly dissociable complexes, X is a relatively long-lived intermediate and C is the stable enzyme–inhibitor complex. This Scheme predicts that dissociation of C by an agent that traps E leads to a mixture of I and I^* . In our case I^* would be eglin c with the Leu-45–Asp-46 peptide bond broken. The trapping agent used in the current study was $\alpha_1\text{PI}$, and the release of eglin c was monitored by electrophoresis. Since the inhibitor has no disulphide bonds [11], I^* should migrate as two low-molecular-mass peptides with different charges. Such a pattern was not observed on the various electrophoregrams. Trace amounts of I^* would of course not be detected by electrophoresis, the more so in that we followed the dissociation reaction only to about 40 % competition. We may nevertheless conclude that the $C \rightleftharpoons X \rightleftharpoons L^* \rightleftharpoons E + I^*$ pathway is not significant in our case. This view is supported by the observation that the Leu-45–Asp-46 peptide bond is intact within the eglin c–subtilisin complex [11]. Also, for enzyme–inhibitor systems that obey the standard mechanism, the $[I^*]/[I]$ ratio at equilibrium is very low at neutral pH [9].

The linear dependence of $k_{\text{obs.}}$ on $[I_0]$ indicates that the eglin–chymotrypsin association apparently does not conform to

the left-hand side of the standard mechanism, i.e. that E and I associate to form directly C (= EI). Several lines of evidence suggest, however, that the association process might be more complex than a simple bimolecular reaction. First, $k_{\text{assoc.}}$ is two orders of magnitude lower than the maximum rate constant ($\approx 5 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$) for a bimolecular diffusion-controlled reaction as estimated by the method of Albert & Hammes [27]. Secondly, the activation energy for the association process at pH 7.4 ($66 \text{ kJ} \cdot \text{mol}^{-1}$) or at pH 5.0 ($78 \text{ kJ} \cdot \text{mol}^{-1}$) is much higher than that for a diffusion-controlled step [28]. The literature describes enzyme-inhibitor systems which follow the $\text{E} + \text{I} \rightleftharpoons \text{EI}^* \rightleftharpoons \text{EI}$ mechanism while having activation energies for association of $44 \text{ kJ} \cdot \text{mol}^{-1}$ [29] or even of $56 \text{ kJ} \cdot \text{mol}^{-1}$ [13]. It must therefore be assumed that the eglin-chymotrypsin association involves an intermediate EI^* which cannot be detected with the current technology, i.e. K_1^* (app.) of eqn. (7) must be higher than the highest inhibitor concentration tested. At pH 7.4 and 5°C we must therefore have $K_1^* \text{ (app.)} \gg 1 \text{ mM}$, i.e. $K_1^* \gg 0.5 \text{ mM}$. The values of K_1^* measured for other enzyme-inhibitor systems range from $65 \mu\text{M}$ to $500 \mu\text{M}$ [12,13,29–32]. From $K_1^* \gg 0.5 \text{ mM}$ one infers that k_2 (Scheme 4a) must be much greater than 2000 s^{-1} . This value is more than 6-fold higher than the highest isomerization rate constant measured for a proteinase-inhibitor system [13], but could represent the rate constant for a fast conformational change of a protein [33]. Thus eglin c and chymotrypsin might react with each other according to a mechanism in which a very loose pre-equilibrium complex EI^* rapidly isomerizes into an extremely stable final complex EI:

Scheme 6

with $k_{-1}/k_1 = K_1^* \gg 5 \times 10^{-4} \text{ M}$, $k_2 \gg 2 \times 10^3 \text{ s}^{-1}$, $k_{-2} = k_{\text{dissoc.}} \approx 1.6 \times 10^{-6} \text{ s}^{-1}$, $k_2/K_1^* = k_{\text{assoc.}} = 4.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $K_1 \approx 4 \times 10^{-13} \text{ M}$.

A striking feature of the eglin c-chymotrypsin association is the very mild effect of pH on $k_{\text{assoc.}}$, which varies by less than 50% below and above neutral pH. To the best of our knowledge, this is the first proteinase-inhibitor system for which $k_{\text{assoc.}}$ is almost independent of pH. The chymotrypsin-pancreatic-trypsin-inhibitor pair associates about 100-fold faster at pH 7.0 than at pH 5.0 and the sigmoidal variation of $k_{\text{assoc.}}$ with pH is described by a titration curve with a pK_a of 7.0 attributable to the ionization of His-57 of the enzyme's active centre [13]. Similar sigmoidal-shaped titration curves have been reported for other systems, $k_{\text{assoc.}}$ being at least 5-fold higher at neutral pH than at pH 4–5 [30,34]. For other systems, the pH-dependency of $k_{\text{assoc.}}$ was a bell-shaped titration curve involving an unprotonated acid and a protonated base of the enzyme in the interaction [29,31,35]. More remarkable is the observation that human pancreatic elastase, another chymotrypsin-like enzyme, associates with eglin c with a $k_{\text{assoc.}}$ that increases 6-fold between pH 5.0 and 8.0 [21].

In an attempt to rationalize the data obtained with the eglin-chymotrypsin system, we have assumed that one partner, i.e. eglin c, is active for association whatever its state of ionization, whereas the other is about 45% active if an acidic group of pK_a 6.3 is fully protonated or if a base with pK_a of 8.1 is fully deprotonated. With these assumptions, the bell-shaped pH-dependency curve could be fitted to the model outlined in eqn. (8). The pK_a of 6.3 might correspond to His-57 of chymotrypsin, whose deprotonation activates the enzyme's catalytic triad. The pK_a of 8.1 is more difficult to rationalize as it is at least one unit

lower than the pK_a of the N-terminal $\alpha\text{-NH}_2$ group of the enzyme which controls the conformation of the active site [36]. We are not unaware that the present model is purely empirical and that others might be suggested.

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