Kinetic evidence for a two-step mechanism for the binding of chymotrypsin to α_1 -proteinase inhibitor

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We have used the proflavin displacement method and a stopped-flow apparatus to measure the rate constant for the binding of 2 μ M-chymotrypsin to 20–125 μ M- α_1 -proteinase inhibitor. The observed pseudo-first-order constant showed a hyperbolic dependence on α_1 -proteinase inhibitor concentration, suggesting a reaction mechanism in which a fast pre-equilibrium ($K = 0.19$ mM) is followed by a first-order formation of the final complex ($k = 252$ s⁻¹).

INTRODUCTION

Human plasma α_1 PI is a 53 kDa glycoprotein formed of a single chain of 394 amino acid residues whose primary structure is known. It is a serine-proteinase inhibitor whose major function is to protect the lung alveolar epithelium against the deleterious action of neutrophil elastase [1,2]. It is the archetype of the so-called serpin superfamily [3].

The proteinase- α_1 PI complexes resist dissociation by denaturing agents such as SDS or urea, suggesting stabilization by covalent bonding. Studies with post-complex α_1 PI showed that peptide-bond hydrolysis occurs at the Met-358–Ser-359 linkage, the so-called active centre of α_1 PI. Accordingly, it has been hypothesized that the enzyme-inhibitor complex is stabilized by a covalent bond between Met-358 and the serine residue of the catalytic triad of the proteinase. Whether the native complex contains this acyl-enzyme bond or whether this bond is formed as a consequence of denaturation remains, however, to be elucidated [1]. The crystal structure of the native complex has not yet been established. In the highly ordered post-complex α_1 PI, Ser-359 is 6.7 nm (67 Å) remote from its original position, suggesting that the inhibitor undergoes a conformational change upon peptide-bond hydrolysis at its bait region [4]. Conformational studies in solution directly demonstrate that proteolytic cleavage of α_1 PI at this region triggers a major structural rearrangement of the molecule with subsequent increase in conformational stability [5].

From a kinetic point of view, α_1 PI behaves like an irreversible inhibitor. When tested at $[E]_0 = [I]_0 = 10$ nM–3 μ M, second-order kinetics are indeed observed for a variety of serine proteinases. The second-order association rate constants ($k_{\text{ass.}}$) range from 48 M⁻¹·s⁻¹ (thrombin) to 6.5 × 10⁷ M⁻¹·s⁻¹ (neutrophil elastase) [6]. However, even the highest of the rate constants is lower than would be expected for a diffusion-controlled process, suggesting that the enzyme + inhibitor association is more complex than a simple bimolecular reaction ($E + I \rightarrow EI$) and probably involves a fast pre-equilibrium followed by

a slow first-order rearrangement of the complex ($E + I \rightleftharpoons EI^* \rightarrow EI$). The present paper is the first report to show that the binding of chymotrypsin to α_1 PI follows such a two-step mechanism.

MATERIALS AND METHODS

Three-times-crystallized bovine α -chymotrypsin (Worthington) was active-site-titrated with *N-trans*-cinnamoyl-imidazole [7]. The gram quantities of α_1 PI required for the stopped-flow experiments were isolated as described previously [8], dialysed against 0.29 M-NaCl/20 mM-Hepes/NaOH buffer, pH 7.4, concentrated up to about 25 mg/ml on an Amicon PM 10 device and clarified by centrifugation. The inhibitor migrated as a single band on SDS/polyacrylamide-gel electrophoresis and inhibited chymotrypsin [6] with a 1:1 molar binding stoichiometry. Gel filtration of a 25 mg/ml α_1 PI solution on a Toya-Soda TSK SW 3000 column connected to a Beckman h.p.l.c. apparatus gave a symmetrical peak composed of fully active inhibitor, suggesting that α_1 PI does not aggregate to a significant extent at high concentrations.

All experiments described below were done at 16 °C in the 0.29 M-NaCl/20 mM-Hepes buffer, pH 7.4. The dissociation constant K_p of the chymotrypsin–proflavin complex (20 ± 0.8 μ M) was measured by differential spectrophotometry at 465 nm [9] in a Cary 118 apparatus. Differential spectroscopy at 465 nm also showed that α_1 PI stoichiometrically displaced proflavin from its complex with chymotrypsin, indicating that the dye and the inhibitor were competing ligands for the enzyme and that the decrease in absorbance at 465 nm ($\Delta\epsilon = 1.7 \times 10^4$ M⁻¹·cm⁻¹) can be used to monitor the chymotrypsin- α_1 PI association. Free or chymotrypsin-bound α_1 PI did not bind proflavin.

Stopped-flow experiments were run on a Durrum–Gibson instrument on-line with a PDP 11 computer. The cell path-length was 1 cm and the dead time about 3 ms. One syringe contained chymotrypsin (E) and proflavin (P) and the other was filled with α_1 PI (I). The final concentrations in the mixing chamber were $[E]_0 = 2$ μ M,

Abbreviation used: α_1 PI, α_1 -proteinase inhibitor.

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Fig. 1. Influence of α_1 PI concentration on k_{obs} for the binding of chymotrypsin to α_1 PI at pH 7.4 and 16 °C

○, Experimental points; —, theoretical curve generated by using eqn. (3) with $k_{+2} = 252 \text{ s}^{-1}$ and $K_{\text{I(app.)}} = 430 \text{ } \mu\text{M}$; ·····, tangent to the theoretical curve.

$[\text{P}]_0 = 24 \text{ } \mu\text{M}$ and $[\text{I}]_0 = 20\text{--}125 \text{ } \mu\text{M}$ (i.e. $[\text{I}]_0 \gg [\text{E}]_0$, $[\text{E}]_0 \ll K_p$ and $[\text{P}]_0 \approx K_p$). Under these conditions, the change in absorbance at 465 nm measures the binding of E and I and the association kinetics are pseudo-first-order [10]. Single exponentials were indeed recorded for the whole range of α_1 PI concentrations and were used to calculate apparent pseudo-first-order rate constants k_{obs} . All k_{obs} values reported in Fig. 1 are averages for six to eight measurements.

RESULTS AND DISCUSSION

For $[\text{E}]_0 = [\text{I}]_0 = 10 \text{ nM}$ [4] or 100 nM [9] the inhibition of chymotrypsin by α_1 PI obeys second-order kinetics characteristic of a bimolecular irreversible association process:

Scheme (1) predicts a linear dependence of k_{obs} on $[\text{I}]_0$ but Fig. 1 shows significant deviation from linearity for $[\text{I}]_0 > 50 \text{ } \mu\text{M}$. The latter is best accounted for by assuming a two-step reaction mechanism:

in which a fast pre-equilibrium between E, I and EI^* ($k_{-1} \gg k_{+2}$) precedes the formation of the final complex EI. From $K_1 = k_{-1}/k_{+1}$ and $K_{\text{I(app.)}} = K_1(1 + [\text{P}]_0/K_p)$ one gets:

$$k_{\text{obs.}} = \frac{k_{+2}[\text{I}]_0}{[\text{I}]_0 + K_{\text{I(app.)}}} \quad (3)$$

an equation that describes a hyperbolic dependence of $k_{\text{obs.}}$ on $[\text{I}]_0$. A plot of $1/k_{\text{obs.}}$ versus $1/[\text{I}]_0$ gave a straight line from which rough estimates of k_{+2} and $k_{\text{I(app.)}}$ were derived. The refined parameters and their confidence intervals were calculated by non-linear-regression analysis using a program fitting the experimental data to eqn. (3). The following values were obtained: $k_{+2} = 252 \pm 33 \text{ s}^{-1}$, $K_{\text{I(app.)}} = 430 \pm 68 \text{ } \mu\text{M}$ and $K_1 = 195 \pm 30 \text{ } \mu\text{M}$. The confidence intervals of k_{+2} and K_1 are reasonably low (13% and 16% respectively). In addition, there is a good fit between the experimental points and the theoretical curve calculated with the above k_{+2} and $K_{\text{I(app.)}}$ values (see Fig. 1). This suggests that the data are reliable, although the highest inhibitor concentration used corresponds only to about one-third of $K_{\text{I(app.)}}$.

This is the first demonstration of a two-step mechanism for the interaction of α_1 PI with a proteinase. The k_{+2}/K_1 ratio ($1.3 \times 10^6 \pm 0.4 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$) compares well with $k_{\text{ass.}}$ measured under second-order conditions at pH 7.5 and 20 °C in the presence of 0.6 M-KCl ($k_{\text{ass.}} = 0.8 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$) [11]

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