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SOYBEAN TRYPSIN INHIBITOR (KUNITZ) IS DOUBLEHEADED

KINETICS OF THE INTERACTION OF α-CHYMOTRYPSIN WITH EACH SIDE

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Summary

Further evidence is presented for the formation of a ternary complex between α-chymotrypsin (EC 3.4.21.1) and soybean trypsin inhibitor as well as between α-chymotrypsin and a preformed complex of soybean trypsin inhibitor and trypsin (EC 3.4.21.1). This is well in agreement with our earlier sedimentation equilibrium studies. We report on different elution patterns of the ternary forms as compared to the inhibitor trypsin complex and the individual components in gel filtration studies. We also demonstrate the decrease of a given chymotryptic activity on a substrate if the solution is mixed with another one containing the preformed stoichiometric inhibitor-trypsin complex. A fourth piece of evidence for the formation of a chymotrypsin-inhibitor-trypsin complex is the appearance of a difference spectrum in absorbance, when chymotrypsin is mixed with the inhibitor-trypsin complex. Inhibition studies with purified inhibitor show that one molecule of inhibitor binds two molecules of α-chymotrypsin, with dissociation constants K_1 about 1 μM and K_2 about 300 nM at pH 8. The site with weaker affinity for chymotrypsin is specifically blocked by stoichiometric amounts of trypsin.

Purification of commercially available preparations of soybean trypsin inhibitor (Kunitz) ('inhibitor') to apparent homogeneity using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and first-order association kinetics with β-trypsin, is achieved by a combination of gel filtration and ion-exchange chromatography. The kinetics of the interaction of chymotrypsin with inhibitor or with inhibitor-trypsin complex were measured in a stopped-flow photometer by following the displacement of proflavine from the active

site of chymotrypsin. A complete reaction scheme is presented with all rates and equilibrium constants as well as their pH-dependence.

Introduction

The interaction of serine proteases with their protein inhibitors has been intensively studied, especially by crystallographic and kinetic methods [1—3]. These efforts have yielded considerable insight into the catalytic mechanism of proteolysis. In the case of trypsin/soybean trypsin inhibitor it was discovered that the enzyme is able to split a reactive peptide bond in the active site of the inhibitor, leading to a 'modified inhibitor' [4,5]. At equilibrium, the ratio of modified to unsplit inhibitor is near unity at neutral pH [4,5]. These observations were later conformed for many other protein protease inhibitors [4—6].

Soybean trypsin inhibitor inhibits α-chymotrypsin (EC 3.4.21.1) several orders of magnitude more weakly than trypsin [7]. De Vonis Bidlingmeyer et al. [8] have shown that α-chymotrypsin binds to the same binding site of the inhibitor as trypsin, and that it also splits the reactive site peptide bond of the inhibitor. We have presented evidence that soybean trypsin inhibitor binds two molecules of chymotrypsin with comparable affinity [9]. Results from sedimentation equilibrium experiments, using an analytical ultracentrifuge, were in agreement with a 1 : 2 stoichiometry but not with a 1 : 1 association of inhibitor with chymotrypsin. In this paper, the purification of commercially available inhibitor preparations to apparent homogeneity will be described. Additional evidence for the existence of a second binding site of the inhibitor for α-chymotrypsin is presented and the kinetics of complex formation at either binding site is given.

Methods and Materials

α-Chymotrypsin. α-Chymotrypsin crystallized 3-times, from Worthington, showed a minor impurity (5% of the total protein) of molecular weight 40 000 when it was subjected to SDS-polyacrylamide gel electrophoresis. Active site titration of the enzyme was performed with a large excess of nitrophenyl-N^2-acetyl-N^1-benzylcarbazate [10] in a stopped-flow photometer. The monophasic acylation kinetics observed indicated kinetic homogeneity of the material. From the reaction amplitude the molar absorption coefficient of α-chymotrypsin at 280 nm (ϵ_{280}) was calculated to be $5.15 \cdot 10^4\ M^{-1} \cdot cm^{-1}$.

Bovine trypsin. Bovine trypsin was purchased from Worthington (lot TRL 33P685). Active site titration of trypsin according to Chase and Shaw [11] gave a molar absorption coefficient of $\epsilon_{278} = 4.6 \cdot 10^4\ M^{-1} \cdot cm^{-1}$. This material was used to block the first binding site of purified inhibitor, using a 10% molar excess of trypsin over inhibitor. β-Trypsin was prepared by a modification [12] of the method of Schroeder and Shaw [13] and contained less than 5% α-trypsin [12].

Soybean trypsin inhibitor. Inhibitor was purchased from Worthington, from Serva and Merck. Purification of these preparations is described below. Proflavine monohydrochloride (purum) was obtained from Fluka (Switzerland)

and 4-nitrophenyl-N^2-acetyl-N^1-benzylcarbazate from Nutritional Biochemicals Corp., Cleveland, OH. All other chemicals (reagent grade) were obtained from Merck.

Difference spectra were measured with a Cary 118 spectrophotometer using a matched pair of tandem cells with a pathlength of 0.483 cm per compartment [12]. Gel filtration studies were carried out at 23° C. A Cary 219 spectrophotometer was used with a flow cuvette for continuous registration.

For equilibrium inhibition curves the concentration of free enzyme was determined from the residual enzyme activity by using α-*N*-(3-carboxypropionyl)-phenylalanine-4-nitroanilide as substrate [16]. 1 ml chymotrypsin-inhibitor solution was added to 1.5 ml substrate solution. Since the lifetime of the inhibitor-chymotrypsin complex is shorter than the deadtime of the enzymatic test (approx. 10 s), the complex decays within that time to a new equilibrium value because of dilution and the presence of substrate. The experimental value K' of the equilibrium constant K = [Enzyme]/[Inhibitor]/[Complex], must therefore be corrected according to

$$K' = K(1 + S_0/K_m) \quad (1)$$

(see Eqn. 11 from Ref. 17), where S_0 denotes the substrate concentration and K_m the Michaelis constant. K_m was determined to 1.6 mM at pH 6, 1.1 mM at pH 7 and 1.0 mM at pH 8.

The interaction of chymotrypsin with inhibitor was also followed by active site titration of the enzyme. Solutions of chymotrypsin-inhibitor complex were rapidly mixed with solutions of the active site titrant 3-nitrophenyl-N^2-acetyl-N^1-benzylcarbazate, by means of a T-shaped connector in a flow-through cuvette. The titrant displaces the inhibitor from the active site of the enzyme and forms the acyl enzyme. The accompanying release of *p*-nitrophenyl was followed in the sample compartment of a double-beam spectrophotometer. The strong spontaneous hydrolysis of the active site titrant ($t_{1/2}$ approx. 60 min at pH 7) was compensated by adding the same concentration of titrant to the reference cuvette [14].

Stopped-flow measurements were carried out with a modified Durrum Gibson apparatus [12] connected on line to a PDP 11 computer.

The kinetics of the interaction of chymotrypsin with inhibitor or with inhibitor-trypsin complex were followed by proflavine displacement from the active site of chymotrypsin, by residual enzyme activity and by active site titration. In the proflavine displacement experiments solutions of chymotrypsin-proflavine complex were rapidly mixed with inhibitor solutions in a stopped-flow photometer. The inhibitor displaces the dye from the active center of the enzyme and this is accompanied by a large change in absorption at 465 nm [14, 15]. Experimental values of second-order-rate constants or equilibrium constants were corrected for the fraction of enzyme occupied by the dye as described earlier [14].

The proflavine displacement method gives very much larger changes in absorption, than the intrinsic absorption difference, (shown in the difference spectrum, Fig. 3) as a means of following the association of the proteins. Its applicability was checked by measuring the association with the natural absorption difference for a few concentrations. No difference in the rate was observed

but there was a lower signal to noise ratio when the intrinsic difference was used.

Results

Purification of inhibitor

Purity of inhibitor was judged by SDS-polyacrylamide gel electrophoresis and by the kinetic pattern observed in the association with β-trypsin. At pH 7.5 the association kinetics of excess inhibitor with trypsin follow a single exponential [19], yielding a second-order-rate constant of $5 \cdot 10^6\ M^{-1} \cdot s^{-1}$. In SDS-polyacrylamide gel electrophoresis all commercial preparations showed impurities of various molecular weights which accounted for up to 50% of the total material. Gel filtration on a Sephadex G-75 column (100 × 5 cm) [20] removed impurities of molecular weight 60 000, 38 000, 16 000 and 12 000. After lyophilization the material was applied to a DEAE-cellulose column (Whatman DE-52) equilibrated with 50 mM Tris-HCl buffer at pH 7.5 and eluted with a linear gradient of NaCl (see Fig. 1A for details). The pooled fractions migrated as a single band in SDS-polyacrylamide gel electrophoresis. However, the association kinetics with β-trypsin were biphasic, with 20% of the total amplitude contributed by a slow phase. The inhibitor was, therefore, rechromatographed on the DEAE-cellulose column eluted with a gradient from 175 mM sodium acetate at pH 5 to 200 mM sodium phosphate at pH 2 (see Fig. 1B). The final material in about 20% yield was kinetically homogeneous in its reaction with β-trypsin. The molar absorption coefficient of the active inhibitor was determined by inhibition of the active site titration of trypsin [11]. A value of $1.7 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ was obtained which is slightly lower than the values used in the literature [19], indicating a higher activity of this preparation.

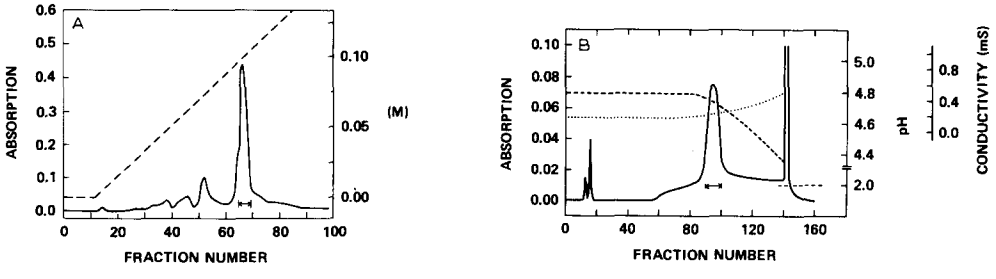


Fig. 1. DEAE-cellulose chromatography of inhibitor. A: Elution by salt gradient at pH 7.5. A Whatman DE-52 cellulose column (29 × 2.5 cm) was equilibrated with 0.05 M Tris-HCl buffer at pH 7.5. 1 g inhibitor, after gel filtration on Sephadex G-75 (see text), was eluted with a linear gradient of potassium chloride (total volume = 2 l, gradient: 0—0.15 M KCl). Elution was followed by absorbance at 280 nm. Fractions of 20 ml were collected at a flow rate of 1 ml/min. Fractions 64—69 were pooled and lyophilized. B: Rechromatography by a continuous gradient of pH and ionic strength. The DEAE-cellulose column was equilibrated with 0.175 M sodium acetate buffer at pH 5.0. 6 mg inhibitor, purified as described in Fig. 1A, were eluted with a linear gradient of 800 ml of 0.175 M CH_3COONa at pH 5 and 800 ml of 0.2 M sodium phosphate at pH 2. The elution was followed by absorption at 280 nm (left ordinate, (———)). The changes in pH (- - - - - -) and in conductivity, measured in mSiemens (· · · · · ·) are indicated at the right ordinate. 15 ml fractions were collected at a flow rate of 40 ml/h.

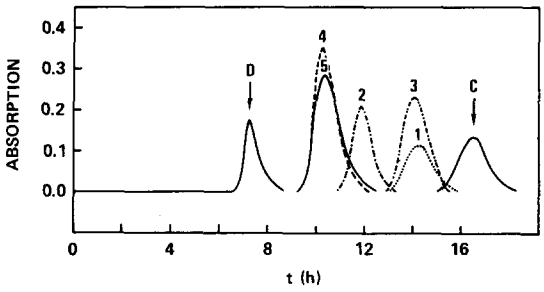


Fig. 2. The formation of two ternary complexes of chymotrypsin and trypsin with inhibitor, are shown by these profiles of five different elutions on the same 2.5 × 95 cm long Sephadex G-100 column operated at room temperature. The same amounts of Blue Dextran (D) and cytochrome *c* (C) were added as reference compounds to each applied sample giving a total volume of 5 ml. (1) 0.5 μmol inhibitor (· · · · · ·) and (2) 0.5 μmol inhibitor-trypsin (· — · — ·) complex were run in 0.1 M potassium phosphate buffer, pH 8. A mixture of (3) 0.5 μmol trypsin and 0.5 μmol chymotrypsin (· · · — —), (4) 0.5 μmol chymotrypsin-inhibitor-chymotrypsin complex (- - - - -) and (5) 0.5 μmol trypsin-inhibitor-chymotrypsin (———) complex were eluted in 2 μM chymotrypsin.

Gel filtration of the ternary complexes

Gel filtration on Sephadex G-100 was used to show the existence of the ternary complexes of inhibitor, with either two molecules of chymotrypsin or with one molecule of trypsin and one of chymotrypsin. Fig. 2 shows the profiles of five separate elutions on the same column. Two well-separated standards, Blue Dextran and cytochrome *c*, were present in each individual separation and were eluted reproducibly at the same position. Therefore, the different positions (1—5) in the elution profile must be due to significantly different molecular weights. Apparent molecular weights of 72 500 and 74 000 can be derived for the trypsin-inhibitor-chymotrypsin and chymotrypsin-inhibitor-chymotrypsin complexes, respectively. These values are not only very close to the theoretically calculated molecular weights, they also suggest that the two complexes have similar shapes. The concentration of the complexes in the applied samples was 100 μM. The buffer contained 2 μM chymotrypsin for elutions 4 and 5 to account for dissociation. The observed stability of the two complexes under the separating conditions of gel filtration is in agreement with their kinetically determined dissociation constants of approx. 1 μM. Chymotrypsin had to be present in the buffer at a concentration of at least 1 μM because of the kinetically-determined high dissociation constants of the complexes of 1.5 s^{-1} and 2.3 s^{-1}. The fairly symmetrical elution profiles of the two complexes in turn confirm the kinetic data that the dissociation constants are not higher than 1 μM. In order to exclude the possibility that this low concentration of chymotrypsin alters the elution profile, the elutions of trypsin and chymotrypsin were also run in this buffer. The fact, that the mixture of trypsin and chymotrypsin is eluted very closely to the elution position of the inhibitor, shows that there is no interaction between the proteases under the conditions of the experiment. The elution profile of the very stable and well-defined inhibitor-trypsin complex is included for comparison.

Absorption difference spectra

Absorption difference spectra were measured with solutions of chymotrypsin

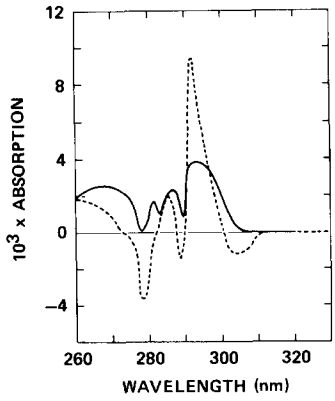


Fig. 3. Absorption difference spectra obtained after mixing chymotrypsin with (a) inhibitor (- - - - -) and (b) inhibitor-trypsin complex (———) in tandem double cells (pathlength 0.438 + 0.438 cm). The concentrations were after mixing (a) [chymotrypsin]$_0$ = 29 μM, [inhibitor]$_0$ = 12.5 μM and (b) [chymotrypsin]$_0$ = 14.5 μM, [inhibitor-trypsin complex]$_0$ = 12.5 μM, in 50 mM Tris-HCl buffer with 0.3 M KCl at pH 7.5.

and inhibitor or inhibitor-trypsin complex for two reasons. First, the existence of an absorption change directly reflects an interaction between the proteins, which is further evidence for the formation of ternary complexes. Second, it can also be used to follow directly the association kinetics of chymotrypsin with inhibitor or with inhibitor-trypsin complex.

Fig. 3 shows the absorption difference spectra obtained after mixing chymotrypsin with inhibitor (a) or with inhibitor-trypsin complex (b). Spectrum (a) is similar in shape to spectrum (b) but about twice as large. The difference of spectrum (b) constitutes direct evidence for an interaction of chymotrypsin with a secondary binding site of the inhibitor, since the classical first site [4,8] is completely blocked by trypsin. Taking into consideration that at the chosen concentrations only 85% of the total inhibitor-trypsin complex is bound to chymotrypsin ($K_2 = 4.7 \cdot 10^{-7}$ M, see below), and correcting for the pathlength of the tandem cuvette, one calculates a value of 400 $M^{-1} \cdot cm^{-1}$ for the difference in molar absorption coefficient between complex and free components. This value is about 4-times smaller than the values found for the interaction of trypsin with this inhibitor [19] or with pancreatic trypsin inhibitor [12], but comparable to the value found for chymotrypsin/pancreatic trypsin inhibitor [14].

Equilibrium inhibition curves

The inhibition of chymotryptic activity by inhibitor was used to demonstrate again the existence of the ternary complexes Tr-I-Ch (trypsin-inhibitor-chymotrypsin) and Ch-I-Ch as well as to determine their stoichiometry and dissociation constants.

Fig. 4 shows a titration of α-chymotrypsin with inhibitor and with inhibitor-trypsin complex at pH 8. The initial slopes (Fig. 4, dotted lines) extrapolated toward the abscissa intercepts * $[I]_0/[Ch]_0 = 0.5$ or $[ITr]_0/[Ch]_0 = 1$. This indi-

* [ ]$_0$ denotes total concentration.

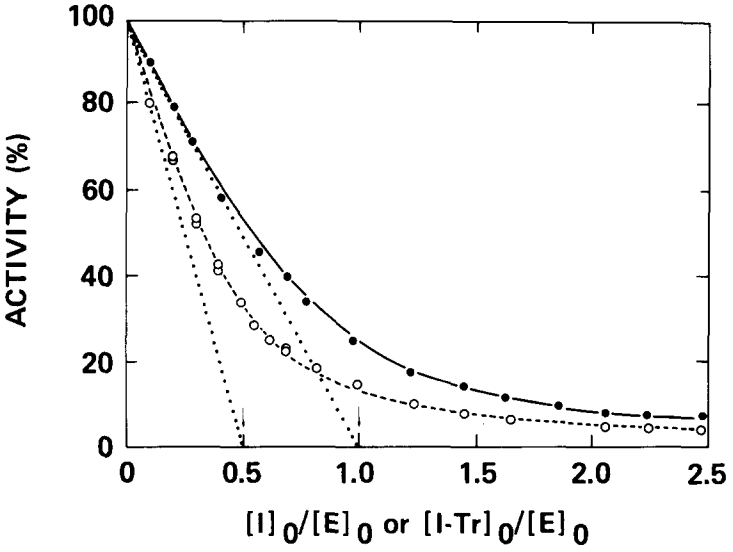
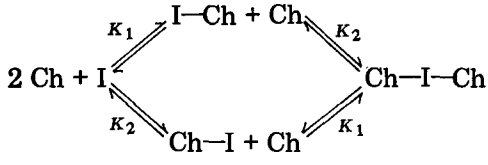


Fig. 4. Titration of chymotrypsin with inhibitor-trypsin complex (●———●) and free inhibitor (○———○) as followed by enzymatic test. 1 ml of a chymotrypsin-inhibitor solution was added to 1.5 ml of a solution of N^{α} (3-carboxypropionyl)-L-phenyl alanine 4-nitroanilide. The final concentrations were [chymotrypsin]$_0$ = 12 μM, [substrate]$_0$ = 4.8 mM, both in 0.05 Tris-HCl buffer/0.3 M KCl/5% dimethylformamide at pH 8. The initial slopes (· · · · · ·) extrapolate toward [I]$_0$/[E]$_0$ = 0.5 or [I-Tr]$_0$ = 1 indicated by the arrows. The fit to the inhibition curve for inhibitor-trypsin complex was calculated according to the law of mass action with K_2 = 270 nM. The fitting curve (- - - - - -) was calculated using K_2 = 270 nM and K_1 = 1.5 μM (all values corrected for the presence of substrate).

cates a stoichiometry of 2 : 1 for the interaction of chymotrypsin with inhibitor and of 1 : 1 for the interaction with inhibitor-trypsin complex. The fit to the inhibition curve for inhibitor-trypsin complex was calculated according to the law of mass action using an equilibrium constant of K_2 = 0.26 μM. The inhibition curve for inhibitor was calculated assuming that the two binding sites are independent. The interaction of chymotrypsin with inhibitor can be described as below



(2)

where the indices of the equilibrium constants denote the interaction with the tryptic site (1) and the second site (2) of the inhibitor, respectively. The free enzyme concentration is obtained by solving the cubic equation derived from scheme (2). The fitting curve was calculated by keeping the value of K_2 = 0.26 μM fixed and adjusting K_1 to 1.2 μM.

Kinetic studies of complex formation

Dissociation of Tr-I-Ch by substrate. It may be concluded from previously described experiments that a Tr-I-Ch complex is formed. Therefore, it should

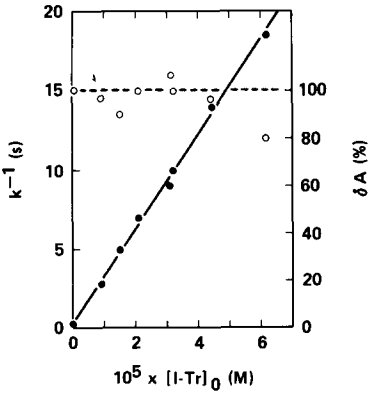


Fig. 5. Inhibition of the acylation of chymotrypsin by inhibitor-trypsin complex. A solution of chymotrypsin and inhibitor-trypsin complex was rapidly mixed with active site titrant. The final concentrations were [chymotrypsin]$_0$ = 3 μM, [active site titrant]$_0$ = 200 μM, in 50 mM sodium phosphate buffer at pH 7.0, containing 200 mM KCl and 2% (v/v) acetonitrile. The left ordinate shows the reciprocal rate of acylation k^{-1} (●———●). The fit of the data to a linearized form of Eqn. 4 gave an equilibrium constant K_2 = 1 μM. The amplitude of the acylation reaction (○———○), right ordinate) was independent of the concentration of inhibitor-trypsin complex.

be possible to dissociate the complex into Tr-I and Ch by the addition of a large excess of substrate for chymotrypsin. Slow kinetic experiments were performed to give further evidence for defined complex formation and the values of dissociation constants.

The decrease of free chymotrypsin concentration after addition of inhibitor-trypsin complex was followed by active site titration of the free enzyme, using the titrant 4-nitrophenyl-N^2-acetyl-N^1-benzylcarbazate [8,10]. After rapid mixing (see Methods and Materials) the titrant displaces the inhibitor-trypsin complex from the active center of chymotrypsin and acylates the enzyme with the release of 4-nitrophenolate. The acyl enzyme is stable [10]. After correction for the spontaneous hydrolysis of the titrant (see Methods and Materials and Ref. 14) the amount of released 4-nitrophenolate corresponds to the amount of acyl enzyme formed. The kinetics of acylation are monophasic. The rate constant depends hyperbolically on the concentration of inhibitor-trypsin complex, whereas the amplitude is constant (see Fig. 5). The simplest scheme in agreement with these observations is

$$\begin{array}{l} \qquad\qquad\qquad\quad N \\ \qquad\qquad\qquad\quad + \\ \mathrm{Tr{-}I{-}Ch} \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} \mathrm{I{-}Tr} + \mathrm{Ch} \\ \qquad\qquad\qquad\quad \downarrow q \\ \qquad\qquad\qquad\quad A + P \end{array} \qquad (3)$$

where N is the active site titrant, A the acylchymotrypsin, P the released 4-nitrophenolate and q the apparent rate constant of acylation. The excess of titrant and of inhibitor-trypsin complex over chymotrypsin was chosen, so that inhibitor binding to chymotrypsin constitutes a fast preequilibrium to the acylation reaction. At [I-Tr]$_0 \geqslant 10$ μM and [N]$_0$ = 100 μM the necessary conditions, i.e., [I-Tr]$_0$ $k_2/q \geqslant 10$, are fulfilled with k_2[I-Tr]$_0 \geqslant 40$ s^{-1} (see

Fig. 8); and q was determined to be 3.3 s^{-1}. The rate equation for formation of the acyl enzyme A is given by

$$\frac{d[A]}{dt} = q[Ch] = q \frac{K_2}{K_2 + [I—Tr]_0} ([Ch]_0 - [A])$$

with the initial condition $[A]_{t=0} = 0$. Formation of A follows a single exponential with the apparent rate constant

$$k = q\, K_2(K_2 + [I\text{-}Tr]_0)^{-1} \tag{4}$$

Fig. 5 shows a semireciprocal plot of k^{-1} vs. $[I\text{-}Tr]_0$. The data are fit by a straight line from which $K_2 = 1$ μM is obtained at pH 7. The amplitude of the acylation reaction is independent of the concentration of inhibitor-trypsin complex, as predicted. This result confirms the mechanism of Eqn. 3.

Stopped-flow kinetics of complex formation. The values of the various rate constants as well as the dissociation constants were obtained from fast kinetic studies using a stopped-flow photometer.

The absorption difference spectra in Fig. 3 are too small to allow reliable measurements of the association kinetics over a sufficiently broad range of concentrations. Therefore, the proflavine displacement method [14] was routinely used.

The second site of the inhibitor was studied by mixing in the stopped-flow photometer chymotrypsin and proflavine in one syringe with inhibitor-trypsin complex and proflavine in the other. Fig. 6 shows a kinetic trace obtained under pseudomonomolecular conditions (final concentration of $[I\text{-}Tr]_0 = 49$ μM. The first-order plot of the kinetics (Fig. 6, right ordinate) is straight, and a relaxation rate of 230 s^{-1} was obtained from these data (corrected for proflavine).

Fig. 7 shows the concentration dependence of the half-time $t_{1/2}$ and of the amplitude δA of the kinetics. The data are in agreement with the bimolecular scheme.

$$Ch + I—Tr \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Tr—I—Ch, \quad K_2 = k_{-2}/k_2 \tag{5}$$

The concentration dependence of $t_{1/2}$ is then given by

$$t_{1/2} = [k_2\sqrt{}]^{-1} \ln \left[1 + \frac{\sqrt{} / Tr—I—Ch_\infty}{1 + \sqrt{} / Tr—I—Ch_\infty}\right] \tag{6}$$

where

$$\sqrt{} = [([Ch]_0 + [I\text{-}Tr]_0 + K_2)^2 - 4\,[Ch]_0\,[I\text{-}Tr]_0\,[I\text{-}Tr]_0]^{1/2}$$

and

$$Tr\text{-}I\text{-}Ch_\infty = \tfrac{1}{2}\,[[Ch]_0 + [I\text{-}Tr]_0 + K - \sqrt{}]$$

When the inhibitor-trypsin complex is in sufficient excess over the enzyme, Eqn. 6 is approximated by

$$t_{1/2} = (\ln 2)\,[k_2[I\text{-}Tr_0] + K_2)]^{-1} \tag{7}$$

In the double logarithmic plot log $t_{1/2}$ vs. log $[I\text{-}Tr]_0$ shown in Fig. 7, the data

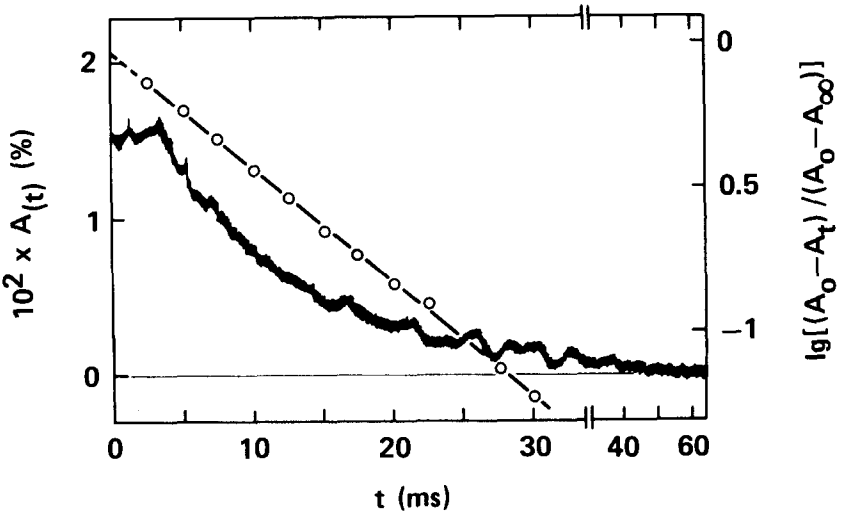


Fig. 6. Association kinetics of chymotrypsin with inhibitor-trypsin complex. The kinetics were followed by proflavine displacement at 465 nm. The total concentrations after mixing in the stopped-flow photometer were: $[\text{chymotrypsin}]_0 = 1.6\ \mu M$, $[\text{I-Tr}]_0 = 49\ \mu M$, $[\text{proflavine}]_0 = 44\ \mu M$ in 0.1 M Tris-HCl buffer containing 0.2 M KCl at pH 7.5 and 20°C. The right ordinate refers to the first-order plot of the kinetics from which an apparent rate constant $k_{app} = 230\ s^{-1}$ was obtained (corrected for the presence of proflavine).

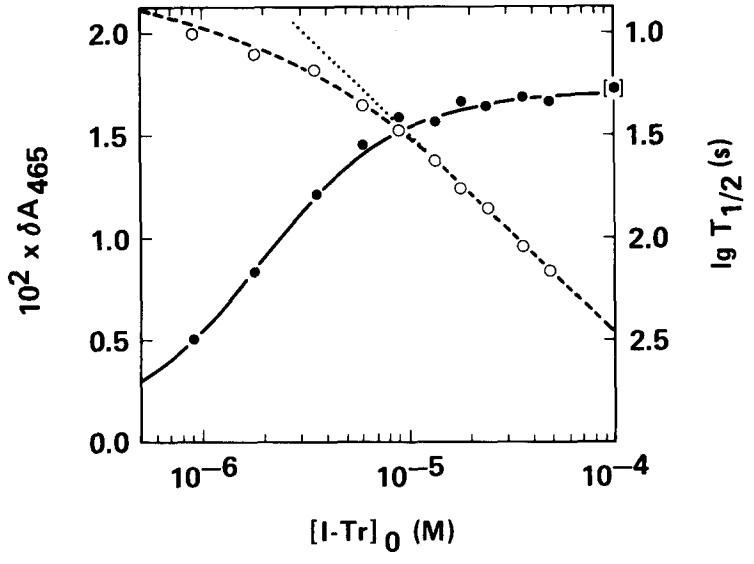


Fig. 7. Concentration dependence of the association kinetics between chymotrypsin and inhibitor-trypsin complex. The amplitude δA_{465} is shown on the left ordinate (●———●). The value at complete saturation ([●]) was measured by mixing the enzyme/proflavine solution with trypsin kallikrein inhibitor in excess. The curve fit to the amplitudes (———) was calculated according to the law of mass action with $K_2 = 0.44\ \mu M$. The reaction half-times ((○———○), right ordinate) were fitted according to Eqn. 6 with $k_2 = 5.4 \cdot 10^6\ M^{-1} \cdot s^{-1}$ and $K_2 = 0.44\ \mu M$ (- - - - - -) all values corrected for proflavine. The dotted line (· · · · · ·) has a slope of (—1) in agreement with Eqn. 7 for $[\text{I-Tr}]_0 \geq K_2$. Experimental conditions were as in Fig. 5.

in the concentration range $[\text{I-Tr}]_0 \geqslant 10$ μM are fitted by a straight line with slope (—1). This indicates that the term K_2 in Eqn. 7 can be neglected and one obtains $k_2 = 5.4 \cdot 10^6\ \text{M}^{-1} \cdot \text{s}^{-1}$. From the fit of the data to Eqn. 6 a value of 0.46 μM was obtained for K_2 (Fig. 7). The amplitudes (Fig. 7) were fitted by the law of mass action with $K_2 = 0.44$ μM. This value can be read from the curve at half saturation where $K_2 = [\text{I-Tr}]_0 - 1/2\ [\text{Ch}]_0$. The amplitude at saturation was determined by mixing the chymotrypsin-proflavine complex with an excess of pancreatic trypsin inhibitor which has a very high affinity for chymotrypsin [14].

When chymotrypsin interacts with both sites of the inhibitor, Eqn. 2 predicts complicated reaction kinetics. In the case of pseudomonomolecular conditions at high saturation, K_1, $K_2 \leqslant [\text{Ch}]_0 \leqslant [\text{I}]_0$, the kinetics are dominated by formation of Ch-I and I-Ch. Then we have

$$\frac{d[\text{Ch}]}{dt} \simeq -(k_1 + k_2)\,[\text{I}]_0\,[\text{Ch}]$$

and the decrease in free enzyme concentration will follow a single exponential decay with a relaxation rate $(k_1 + k_2)\,[\text{I}]_0$. At pH 7.5, a value of $7.1 \cdot 10^6\ \text{M}^{-1} \cdot \text{s}^{-1}$ was obtained for $k_1 + k_2$. Using $k_2 = 5.4 \cdot 10^6\ \text{M}^{-1} \cdot \text{s}^{-1}$, a value obtained for the association of chymotrypsin with inhibitor-trypsin complex, one calculates $k_1 = 1.7 \cdot 10^6\ \text{M}^{-1} \cdot \text{s}^{-1}$.

pH Dependence

In order to see how comparable the two binding sites on the inhibitor are, the interaction of chymotrypsin with inhibitor and with inhibitor-trypsin complex was studied in the pH range from pH 6 to 9.5 by enzymatic test and proflavine displacement. The experiments with inhibitor-trypsin complex yield direct information on the second site of the inhibitor. The parameters for the first (tryptic) site are again obtained by comparison of the data for inhibitor with that for inhibitor-trypsin complex. The results are shown in Fig. 8. The dissociation constants K_1 and K_2 show a significant pH dependence with minima at slightly basic pH. The affinity of chymotrypsin for the first (tryptic) site is somewhat lower than for the second site.

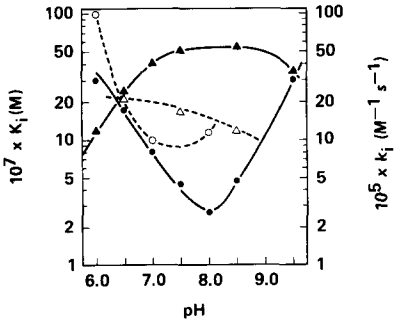
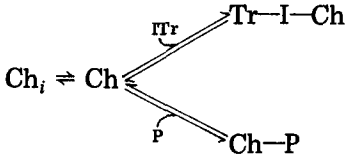


Fig. 8. pH dependence of the equilibrium dissociation constants K_1 (○———○) and K_2 (●———●) and of the association rate constants k_1 (▲———▲) and k_2 (△———△). The buffers were: pH 6—7: 50 mM sodium phosphate, pH 7.5—8.5: 0.1 M Tris-HCl and pH 9—9.5: 0.1 M glycine-NaOH, all containing 0.2 M KCl.

The association rate constant k_2 increases with increasing pH and reaches a plateau at neutral pH. The plateau value, $k_2 \simeq 5 \cdot 10^6\ M^{-1} \cdot s^{-1}$, approaches the limit expected for a diffusion controlled association [22]. A similar pH profile was observed for the association rate constant of chymotrypsin with pancreatic trypsin inhibitor. The pH dependence of k_1 is weak and k_1 is smaller than k_2.

In the pH range pH $\geqslant$ 9.5 and pH $<$ 6 the binding kinetics of inhibitor-trypsin complex and of inhibitor to chymotrypsin became biphasic. A fast decrease in absorption was followed by a slow and smaller increase. The fast phase again represented complex formation between inhibitor and chymotrypsin (compare Fig. 6). The slow increase had a half-time of 1—2 s, independent of inhibitor concentration, whereas the amplitude showed a pronounced maximum as a function of [inhibitor]. These observations are in agreement with a conformational equilibrium at this pH between two forms of chymotrypsin, i.e., an active form Ch and an inactive, non-binding form Ch_i [24].

The association kinetics of chymotrypsin with the inhibitor-trypsin complex in the presence of proflavine are then described by the scheme



(9)

where P denotes proflavine and Ch-P the chymotrypsin-proflavine complex. After mixing, inhibitor-trypsin complex rapidly binds to the active chymotrypsin thus perturbing the conformation equilibrium. In the slow phase [25] active enzyme is formed from Ch_i and partitions into the complexes with I-Tr and with proflavine. Since the change in absorption is proportional to the concentration of chymotrypsin-proflavine complex, it is obvious that the slow phase will disappear if the freshly formed active enzyme goes predominantly into the inhibitor complex. This explains why the slow interconversion between the two forms of chymotrypsin was not observed in our earlier work with pancreatic trypsin inhibitor [14]. Pancreatic trypsin inhibitor is indeed a much stronger inhibitor of chymotrypsin than soybean trypsin inhibitor. In comparison to the data of Fersht [24] the appearance of the inactive form of chymotrypsin is shifted by 1 unit towards higher pH values. A similar shift was observed by Bösterling and Engel [23] in their study on complex formation between fluorescent labeled derivatives of pancreatic trypsin inhibitor and chymotrypsin.

Discussion

Soybean trypsin inhibitor binds two molecules of chymotrypsin with comparable affinity. Direct evidence for the stoichiometry stems from the initial slopes of the titration curves of chymotrypsin with inhibitor and from the gel filtration studies. One of these sites is the trypsin-inhibiting site. De Vonis Bidlingmeyer et al. [8] have clearly shown that chymotrypsin specifically splits and resynthesizes the same reactive site peptide bond of the inhibitor as trypsin does, i.e., the Arg^{63}-Ile^{64} peptide bond. They found a dissociation constant in

the μM range for the interaction of chymotrypsin with this site on the inhibitor [8]. We have therefore blocked this site on the inhibitor by a stoichiometric amount of trypsin. The resulting inhibitor-trypsin complex which is very stable (life-time approx. 20 h [5]), forms a 1 : 1 complex with chymotrypsin, with a dissociation constant smaller than 1 μM. It is therefore clear that chymotrypsin specifically interacts with two different sites of soybean trypsin inhibitor. There are early reports in the literature that this inhibitor inhibits more than one molecule of chymotrypsin [25], in which the specifity and strength of the interaction of chymotrypsin with the second (non-tryptic) site of the inhibitor remained in doubt [8].

When the interaction between chymotrypsin and inhibitor was analyzed it became apparent that the contributions of the two inhibitor sites were difficult to separate. The equilibrium measurements showed that chymotrypsin had a comparable affinity for either inhibitor site. From the stopped-flow experiments it was seen that the respective second-order-rate constants, k_1 and k_2, were of the same order of magnitude. A physical-chemical characterization of site 2 was attempted by blocking the first site of the inhibitor with a stoichiometric amount of trypsin. The underlying assumption is that trypsin bound at site 1 does not profoundly alter the characteristics of site 2. This assumption seemed justified by the following results: First, the inhibitor-trypsin complex formed a 1 : 1 complex with chymotrypsin. Second, the parameters for this interaction, K_2 and k_2, were always within the limits estimated for the second inhibitor site from the interaction of chymotrypsin with inhibitor.

The parameters which were obtained by comparing the interactions of chymotrypsin with inhibitor-trypsin complex and with inhibitor are listed in Table I. The parameters for the interaction of trypsin with inhibitor are included for comparison [5,26]. It is noted that chymotrypsin interacts faster and slightly more strongly with the second site of the inhibitor. The life-time of the inhibitor-chymotrypsin complexes is less than a second. This is several orders of magnitude shorter than the life-times of related protease-inhibitor complexes [5,27]; for example for the trypsin-inhibitor complex Laskowski and co-workers determined a life-time of 17 h [5]. Comparison of the data in

TABLE I

EQUILIBRIUM AND KINETIC PARAMETERS OF THE INTERACTION OF SOYBEAN INHIBITOR WITH α-CHYMOTRYPSIN AND β-TRYPSIN AT pH 7.5

K = [Ch] × [I]/[C] = dissociation constant; k_+, k_- association, dissociation rate constant.

Inhibitor binding site	Enzyme	K (M)	k_+ ($M^{-1} \cdot s^{-1}$)	k_- (s^{-1})
Tryptic (first)	α-chymotrypsin	$9.0 \cdot 10^{-7}$	$1.7 \cdot 10^{6}$	1.5 *
Second		$4.5 \cdot 10^{-7}$	$5.0 \cdot 10^{6}$	2.3 *
Tryptic	β-trypsin	$\sim 3.0 \cdot 10^{-12}$ **	$\sim 5.0 \cdot 10^{6}$ **	$1.6 \cdot 10^{-5}$ **
Second		($>1 \cdot 10^{-4}$) ***		

* Calculated from $k_- = K \times k_+$.

** From Ref. 5, K calculated from $K = k_-/k_+$.

*** See Discussion.

Table I shows that trypsin binds five orders of magnitude more tightly to the inhibitor (site 1) than chymotrypsin does. As observed with many related protease-inhibitor complexes [27] this difference in affinity is reflected in the life-times of the complexes, whereas the association rate constants differ only a little. A very weak interaction was observed between trypsin and the second site of the inhibitor. At concentrations around 100 μM, inhibitor-trypsin complex displaced proflavine from the active center of trypsin (data not shown) and a dissociation constant of 200—500 μM was estimated for this interaction.

The location of the second binding site on the inhibitor molecule is not yet known. Attempts to identify it by specific enzymatic cleavage of an eventual reactive peptide bond located in the binding site [4] have failed. Incubation of inhibitor with chymotrypsin leads to hydrolysis of the Arg^{63}-Ile bond located at site 1, as discovered by Laskowski and co-workers [8]. The hydrolysis of the inhibitor is much slower than the time course of our experiments (Table I). Therefore, change in the inhibitor has not been taken into consideration. In our hands this is followed by slow hydrolysis of several other peptide bonds, finally leading to digestion of the inhibitor. However, it is known that the inhibitor contains a further cleavable bond, i.e., Met^{64}-Leu, which is specifically hydrolyzed by subtilisin [28]. This bond is located at the surface of the molecule, opposite the Arg^{63}-Ile bond in the active center of site 1 (tryptic site) [3]. A steric interference of trypsin bound at site 1 with chymotrypsin bound at the site containing the Met^{84}-Leu bond seems to be excluded. The region around the Met^{84}-Leu bond, therefore, is a plausible candidate for the second binding site of the inhibitor.

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