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Stopped-Flow Kinetics of the Resynthesis of the Reactive Site Peptide Bond in Kallikrein Inhibitor (Kunitz) by β-Trypsin†

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ABSTRACT: Modified kallikrein inhibitor (I*), in which the reactive site peptide bond Lys-15–Ala-16 is split, reacts with β-trypsin (E) according to E + I* ($k_x = 10^5\ M^{-1}\ s^{-1}$) $\rightleftharpoons$ ($k_{-x} = 0.35\ s^{-1}$) X ($k_c = 10^{-2}\ s$) $\rightleftharpoons$ ($k_{-c} = 3 \times 10^{-10}\ s^{-1}$) C (all rate constants at pH 7.5 and 23 °C). In the stable complex C the peptide bond is reestablished and the carboxyl carbon of Lys-15 forms a tetrahedral adduct with Ser-195 of the enzyme. The precomplex X is characterized by an equilibrium constant $K_x = k_{-x}/k_x = 3.5 \times 10^{-6}$ M and an enthalpy of formation of 1.6 kcal/mol. Activation energies of k_x and k_c are 10 kcal/mol. The pH dependencies of K_x, k_x, k_c and of the overall rate constant $k_{on}^* = k_c/K_x$ were measured. For k_{on}^* a bell-shaped pH profile with a maximum at pH 6.8 was observed. With I*-OMe in which the carboxyl group of Lys-15 is esterified by methyl alcohol, the intermediate X was not detected and the rate of C formation was 100-fold faster than in the case of I*. The complex formation with I*-OMe was very similar with that of virgin inhibitor in which the reactive peptide bond is intact. In both cases the pH profile exhibited a plateau in the range of 8–10 and a steep decrease at low pH. The comparison of the data for I*-OMe and I* suggests that in the intermediate complex X the peptide bond is still open and neither the tetrahedral nor the acyl adducts with Ser-195 are formed. For fast formation of the first tetrahedral intermediate, the carboxyl group of Lys-15 must be either protonated or esterified.

Because of the detailed x-ray crystallographic knowledge the interaction of protein inhibitors with serine proteases constitutes a well-defined example of protein–protein interactions. Furthermore, the mechanism of interaction resembles the catalytic process in which a susceptible peptide bond in the reactive site of the inhibitor is split. The normally very stable

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complexes of enzyme and inhibitor contain the susceptible carboxyl group in a tetrahedral state with the reactive peptide bond intact (Huber et al., 1974, 1975; Janin et al., 1974; Sweet et al., 1974).

Most kinetic studies deal with the formation of the complex from virgin inhibitor (for reviews, see Finkenstadt et al., 1974; Lazdunski et al., 1974). Studies of the reaction of the enzyme with modified inhibitors, in which the reactive peptide bond is open, are of considerable interest. In this case complex formation is accompanied by a resynthesis of the peptide bond and must proceed via another sequence of catalytic steps including the formation of the acyl intermediate. So far pertinent results are available only for the reaction of modified soybean trypsin inhibitor with β-trypsin (Luthy et al., 1973). Some preliminary data on the interaction of β-trypsin with the much smaller and chemically different trypsin kallikrein inhibitor in its modified form were presented by Quast et al. (1975, 1976). The present report gives a detailed description of the kinetics of this interaction.

Experimental Section

Materials

Virgin and Modified Inhibitors. Virgin trypsin kallikrein inhibitor I[1] was a kind gift of Bayer AG, Wuppertal. It was homogeneous in gel filtration on Sephadex G-75 and was used without further purification. It was converted to the modified inhibitor I* /1/ according to Tschesche et al. (1974) and Jering & Tschesche (1974a,b, 1976a). The molar absorptivity coefficients were determined from the stoichiometry of trypsin inhibition. Trypsin concentrations were obtained by active site titration with NPGB (Chase & Shaw, 1967). Equilibrium values of inhibition were established instantaneously for I but for I* final values were reached only after 1.5 h. The absorptivity coefficients determined in this way were $\epsilon_{278} = 5.4 \times 10^3$ M^{-1} cm^{-1} for I and 6.1×10^3 M^{-1} cm^{-1} for I*. Since the inhibition test accounts only for active inhibitor, the higher value found for I* as compared with that of I may indicate that the I* preparation contained about 10% inactive protein. All concentrations were based on absorptivity coefficients as determined by inhibition of trypsin and therefore correspond to active inhibitor. Des-(Ala^{16}-Arg^{17}) inhibitor was prepared as described by Jering & Tschesche (1974a,b, 1976a,b).

Modified Inhibitor Esterified with Methanol. I*OMe /1/ was prepared by stirring 15 mg of I* in 0.1 N methanolic HCl at room temperature for 24 h. The solution was filtered, evaporated to dryness, and redissolved in absolute methanol and again brought to dryness. Redissolution and drying were continued until the solution was free of chloride. The residue was then dissolved in 5 mL of water and lyophilized.

The product was characterized by inhibition of trypsin, resistance to carboxypeptidase B, and inactivation by aminopeptidase K. Trypsin (13 μM) was incubated with I*-OMe or I* (3–7 μM) for 1, 10, 15, 20, 30, 360 min and 24 h. The free enzyme was then determined with the substrate BANI /1/ according to Fritz et al. (1970). For I*-OMe final values were obtained after 1-min incubation, while complex formation for I* took 6–24 h. Comparing final values the activity of the I*-OMe preparation corresponded to 70 to 75% of the activity of I*. The molar absorptivity coefficient based on inhibitory activity was $\epsilon_{278} = 7 \times 10^3$ M^{-1} cm^{-1}.

Bovine carboxypeptidase B (EC 3.4.12.3; Merck) was used to demonstrate *O*-methyl ester formation at the Lys-15 carboxyl group of I*. The activity of the enzyme preparation was 60 activity units/mg as defined by the test with hippuryl-L-arginine (Wolff et al., 1962). The enzyme cleaves only C-terminal basic amino acid residues having a free carboxylate group. Failure to inactivate I*-OMe therefore indicates formation of the ester. Stock solutions of 0.16 mg of I* or I*-OMe in 40 mL 0.2 M Tris buffer, pH 7.8, containing 0.1 M NaCl, were prepared. Two millilites of the stock solution were mixed with 0.1 mL of a carboxypeptidase solution with 20 activity units/mL and incubated at room temperature for 1, 5, 10, 20, 40, 80, 120, or 240 min. The reaction was stopped by addition of 20 μL of 3 M HCl and the enzyme was denatured by boiling for 3 min. Prior to the inhibition test, the pH was readjusted to 7.8 by addition of 0.22 mL of 0.04 M Tris buffer, pH 9. The inhibitor activity was again tested by inhibition of trypsin (Fritz et al., 1970) with 24-h incubation in the case of I* and 10-min incubation for I*-OMe. I* was inactivated with a half-time of 2 min, whereas half of the I*-OMe was still active after 120 min. This demonstrates that the carboxypeptidase group of Lys-15 is esterified which protects this residue against carboxypeptidase action. The slow inactivation of I*-OMe may be explained by the side activities of the enzyme preparation or by secondary reactions.

Aminopeptidase K from *Tritirachium album* Limber (EC 3.4.11.1; Merck) was used to inactivate I*-OMe by removal of Ala-16 in order to demonstrate that the susceptible peptide bond between Lys-15 and Ala-16 was open. A resynthesis of this bond during esterification should lead to a product which cannot be inactivated by aminopeptidase. The enzyme (0.058 mg) was added to solutions of 1 mg of I*-OMe or I* in 0.31 mL of 0.025 M sodium diethyl barbiturate buffer, pH 8.3. The total volume was adjusted to 0.46 mL and the mixture was incubated at room temperature for 5, 15, 30, 60, 120, 180 min and 7 h. The reaction was stopped by addition of 20 μL of 3 M HCl. The inhibitory activity against trypsin was determined as described above. Inactivation of I* and I*-OMe proceeded at the same rate with a half-time of about 2 min. The residual inhibitory activity was 3%.

After cleavage of the disulfide bonds in I*-OMe two peptide chains were obtained demonstrating the presence of an open peptide bond. I*-OMe (2 mg) was oxidized with performic acid at −7 °C for 2.5 h according to Hirs (1958). The material was lyophilized after 150-fold dilution with ice–water. It was dissolved in 1 mL of 0.01 M sodium borate buffer, pH 8.6, and placed on a column (0.8 × 60 cm) of CM-Sephadex C-25 equilibrated with the same buffer. The column was eluted with a linear gradient of 0–0.3 M NaCl at a rate of 30 mL/h. Two peptides were obtained and analyzed on the amino acid analyzer after desalting on Bio-Gel P-2. The amino acid composition of the peptides corresponded to the peptide fragments containing residues 1 to 15 and 16 to 56.

I*-OMe proved to be stable at pH values lower than 8. At higher pH I*-OMe seems to hydrolyze slowly to I*. The half-time of hydrolysis at pH 8.0 in 0.025 M Tris buffer containing 0.1 M NaCl was estimated to be 120 min using the carboxypeptidase assay.

Trypsin. Bovine β-trypsin (EC 3.4.21.4), was prepared from crystallized, salt-free trypsin (Worthington and Merck) according to the procedure of Schroeder & Shaw (1968) as modified by Luthy et al. (1973). The β-trypsin preparations

[1] Abbreviations used: I, virgin trypsin kallikrein inhibitor (Kunitz) from bovine pancreas; I*, modified inhibitor in which the reactive peptide bond Lys-15–Ala-16 is open; I*-OMe, I* with the Lys-15 carboxyl group and probably all other carboxyl groups esterified by methyl alcohol; BANI, sodium benzoyl-D,L-arginine-4-nitroanilide hydrochloride; NPGB, 4-nitrophenyl-4-guanidinobenzoate hydrochloride; Tris, tris(hydroxymethyl)aminomethane; DMF, *N,N*-dimethylformamide; $[\ ]_0$, total concentration; $NaDodSO_4$, sodium dodecyl sulfate.

contained less than 5% α-trypsin as judged from overloaded NaDodSO$_4$ gels and from rechromatography on Sephadex SP-50. The same result was obtained from the kinetics of the reaction with NPGB (Hruska et al., 1969) at pH 7. 5. From the active site titration at pH 8.3 according to Chase & Shaw (1967) a molar absorption coefficient of $\epsilon_{278} = 3.9 \times 10^4$ M^{-1} was obtained.

Proflavin was purchased from Fluka, Buchs, Switzerland. All other chemicals were of reagent grade (Merck, Serva).

Methods

Stopped-flow measurements were carried out with a Durrum Gibson stopped-flow photometer (dead time $\approx$ 2.5 ms, 1-cm pathlength) modified by Dr. G. Haenisch in our department. An optical reference system was incorporated and the signal and reference photomultipliers were connected to a logarithmic amplifier, the output of which was directly proportional to the change in absorbance. These modifications improved the signal-to-noise ratio by more than 3. The data were stored with a transient recorder (Datalab DL 905) which was connected on line to a PDP 11/40 computer via an interface designed by Dr. C. Paul. External triggering of the transient recorder allowed sampling at frequencies as low as 0.5 s^{-1} which allowed for measurements of slow phases with half-times up to 500 s. About eight measurements of each concentration were averaged and analyzed as described in the results.

The small intrinsic signal allowed stopped-flow experiments only at protein concentrations higher than 2×10^{-5} M. Therefore the proflavine displacement method (Quast et al., 1974) was employed. All association rate constants and equilibrium binding constants which were derived by this method were corrected by multiplication of the experimental value with the factor $1 + ([P]_0/Q)$ according to Quast et al. (1974), where Q is the dissociation equilibrium constant of the proflavin β-trypsin complex and $[P]_0$ the total proflavin concentration. The correction factor which ranges from 1 to 1.5 accounts for the fraction of enzyme occupied by proflavin. The values of Q were determined to be 1.8×10^{-4} M (pH 5), 1.2×10^{-4} M (pH 6), 1×10^{-4} M (pH 7–8), 1.1×10^{-4} M (pH 9), and 1.66×10^{-4} M (pH 10). The value at pH 7 agreed well with that of Guillain & Thusius (1970). The difference of the molar absorptivity coefficient between bound and free proflavin at 465 nm equalled 1.6×10^4 M^{-1} cm^{-1}. The association reaction of I* with β-trypsin was also followed by addition of the trypsin reactive site titrant NPGB /1/ at various times during the time course of the association reaction. The liberation of p-nitrophenol from NPGB was monitored at 405 nm.

For kinetic control dissociation experiments (Laskowski et al., 1971; Laskowski & Sealock, 1971), 1 mL of 2×10^{-5} M I* and 1 mL of 2×10^{-5} M β-trypsin in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.2 M KCl and 0.05 M $CaCl_2$ were mixed at time $t = 0$ and allowed to react during time t. Then the pH was dropped to 1.8 by addition of 10 μL of 5 M HCl. After adjustment to pH 3, this solution was applied to a Sephadex G-75 column (1.4 × 38 cm) equilibrated at pH 3 with 0.001 M HCl. The inhibitor containing fractions were pooled and concentrated in a small Amicon Diaflo yielding 3.5–4 mL of a 5×10^{-6} M inhibitor solution. The amount of virgin inhibitor in each sample was determined by the displacement of proflavin from chymotrypsin with an error of ±3% from the amplitudes and the reaction half-times. At the concentrations used, reaction of I* with chymotrypsin is negligible within the measuring time (<50 s). The total inhibitor content I + I* of the solution was determined by its inhibitory effect on the tryptic cleavage of BANI. The reliability of the determination of I/(I + I*) was checked by analyzing pure I, pure I*, and mixtures with known composition and was found to be better than ±10%.

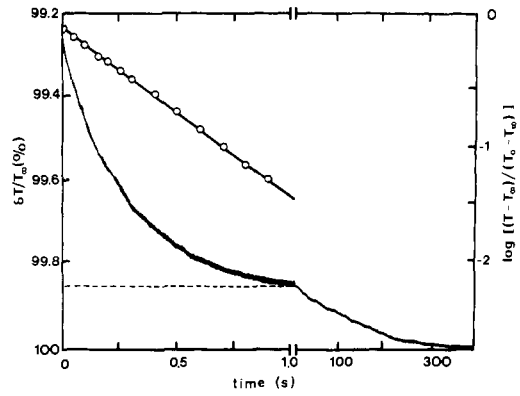

FIGURE 1: Kinetics of the association of β-trypsin with I*. The normalized change in transmittance $\delta T/T_\infty$ at 465 nm resulting from proflavin displacement was recorded. The concentrations in the stopped-flow cell were $[\text{trypsin}]_0 = 2.2 \times 10^{-6}$ M, $[I^*]_0 = 2.1 \times 10^{-5}$ M, $[\text{proflavin}]_0 = 2.3 \times 10^{-5}$ M, in 0.2 M triethanolamine buffer (pH 7.8) containing 50 mM $CaCl_2$ at 22.5 °C. The straight line (right ordinate) is a first-order plot of the fast phase with a pseudo-first-order rate constant of 4 s^{-1}.

The error limits for the parameters derived from fitting theoretical curves to experimental kinetic data were obtained by comparing curves calculated with variations in these parameters. When values were calculated from the fitting parameters, propagation of errors was computed according to standard rules. Difference spectra were measured with a Cary 118 spectrophotometer equipped with thermostated cellholders as described elsewhere (Quast et al., 1974, 1975).

Results

Kinetic Observations and Reaction Mechanism. Figure 1 shows the reaction kinetics of β-trypsin with modified inhibitor in excess ($[I^*]_0 \gg [E]_0$) as measured in the stopped-flow photometer with the proflavine displacement method (Quast et al., 1974). In these experiments, the competitive trypsin inhibitor proflavin (Guillain & Thusius, 1970) is displaced from the enzyme by I*. Two phases are seen which are separated on a time scale by a factor of 100. This observation suggests that E and I* react via an intermediate X to a complex C

$$\mathbf{E} + \mathbf{I}^* \underset{k_{-x}}{\overset{k_x}{\rightleftharpoons}} \mathbf{X} \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} \mathbf{C} \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} \mathbf{L} \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} \mathbf{E} + \mathbf{I} \qquad (1)$$

An alternative mechanism in which X is a nonproductive complex, X $\rightleftharpoons$ E + I* $\rightleftharpoons$ C, leads to an increase of the half-time of the slow phase with increasing concentration of the reaction in contrast to the experimental observation (see next paragraph). We will show that C can be identified with the stable trypsin–inhibitor complex, which is known to be formed also from virgin inhibitor I and the enzyme E (Lazdunski et al., 1974; Finkenstadt et al., 1974). For completeness this known part of the mechanism including the Michaelis pre-complex L is printed in light face in eq 1 and the nomenclature suggested by Finkenstadt et al. (1974) was used. Because of the extremely small value of k_{-2}, the dissociation of C into E and I can be neglected in the present study except for the kinetic controlled dissociation experiments at pH 2.

The fact that the amplitudes of the two phases in Figure 1 are of comparable magnitude whereas the respective half-times differ by more than a factor of 100 proves that k_{-x} is much larger than k_c (for a detailed discussion, see Quast et al., 1974; Luthy et al., 1973). Therefore the intermediate X is in equi-

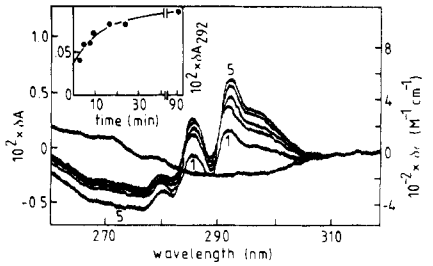

FIGURE 2: Slow build-up of an absorption difference spectrum following mixing of β-trypsin with I*. The first compartments of two tandem cells were filled with 1 mL of a 2.6×10^{-5} M β-trypsin solution and the second ones with 1 mL of 2.6×10^{-5} M solution of I*, both in 0.05 M Tris buffer (pH 8.5) containing 0.2 M KCl and 0.05 M $CaCl_2$. After recording the baseline, the contents of the compartments of the sample cell were mixed and spectra were recorded after (1) 50, (2) 210, (3) 450, (4) 845, and (5) 5400 s. The left and right ordinates show the difference in absorbance δA and in molar absorptivity $\Delta\epsilon$, respectively. Insert: Time course of the change in absorbance δA_{292} as obtained from the difference spectra. The fitted curve was calculated according to eq 8 with $K_x = 1.25 \times 10^{-5}$ M and $k_c = 7 \times 10^{-2}$ s^{-1} (parameters taken from Figure 6).

librium with the free reactants on the time scale of the X → C reaction; e.g., the first reaction step is a fast preequilibrium of the second. The equilibrium constant for the formation of X is

$$K_x = \frac{k_{-x}}{k_x} = \frac{[E][I^*]}{[X]} \tag{2}$$

Under the condition $[E]_0 \ll K_x$ a single step reaction is expected for the reaction of E with I*, the rate constant of which is defined as

$$k_{on}^* = k_c/K_x \tag{2a}$$

in analogy to

$$k_{on} = k_2/K_L \tag{2b}$$

Introduced by Finkenstadt et al. (1974) for the corresponding overall rate constant of the association with the virgin inhibitor. The slow phase shown in Figure 1 could also be followed by the formation of an intrinsic absorption difference spectrum after mixing I* and β-trypsin (see Figure 2). This process is finished after 90 min (Figure 2, insert).

*Concentration Dependence of the Association of β-Trypsin with Modified Inhibitor I**. Equimolar solutions of enzyme and modified inhibitor were mixed in the presence of a constant concentration of proflavin. A fast and slow phase were seen in the entire concentration range. The amplitude of the fast phase $\delta A_1 = \alpha[X]$ is proportional to the amount of intermediate formed. In order to eliminate α, the association of the β-trypsin with virgin inhibitor I in slight excess was measured under identical conditions. The latter is an irreversible one step reaction and has therefore the amplitude $\delta A_0 = \alpha[E]_0$ ($[E]_0 \leq [I]_0$). The normalized amplitude of the fast phase for ($[E]_0 = [I^*]_0$) is

$$\frac{\delta A_1}{\delta A_0} = \frac{[\bar{X}]}{[E]_0} = \frac{K_x}{4[E_0]}\left[\sqrt{1 + 4[E]_0/K_x} - 1\right]^2 \tag{3}$$

where $[\bar{X}]$ denotes the equilibrium concentration of X as calculated from eq 2 with $[E]_0 = [I^*]_0$. Figure 3 shows that the experimental values follow the concentration dependence predicted by eq 3 (left ordinate). From the curve fit $K_x = (3.5 \pm 0.3) \times 10^{-6}$ M was obtained. The concentration dependencies of the reaction half-times of the fast and the slow phase, $T_{1/2}(1)$ and $T_{1/2}(2)$, are also shown in Figure 3 (right ordinate). $T_{1/2}(1)$ exhibits the concentration dependence for the

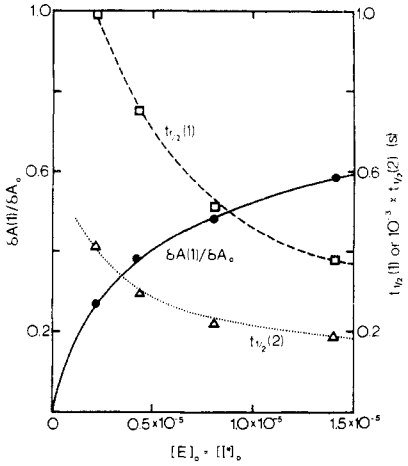

FIGURE 3: Concentration dependence of the association of β-trypsin with I* at equimolar concentrations. The normalized amplitude $\delta A(1)/\delta A_0$ of the fast phase (●) is shown at the left ordinate. Each experimental point is the average of at least six measurements. The curve connecting these points is calculated according to eq 3 with $K_x = 3.5 \times 10^{-6}$ M. The concentration dependence of the half times of the fast phase $T_{1/2}(1)$ (□) and of the slow phase $T_{1/2}(2)$ (△) are shown at the right ordinate. The experimental data are fitted according to eq 4 (- - -) and 9b (• • •) with $K_x = 3.5 \times 10^{-6}$ M; $k_x = 1 \times 10^5$ M^{-1} s^{-1} and $k_c = 1 \times 10^{-2}$ s^{-1}. All solutions in 0.2 M triethanolamine buffer (pH 7.8) containing 50 mM $CaCl_2$; $T = 22.5$ °C; $[\text{proflavin}]_0 = 3 \times 10^{-5}$ M.

reversible reaction E + I* ⇌ X with $[E]_0 = [I^*]_0$:

$$T_{1/2} = \frac{1}{k_x R} \ln\left[1 + \frac{R}{[\bar{X}] + R}\right] \tag{4}$$

with $R = \{K_x(K_x + 4[E]_0)\}^{1/2}$. A good fit was obtained with $K_x = (3.5 \pm 0.3) \times 10^{-6}$ M and $k_x = (1 \pm 0.1) \times 10^5$ M^{-1} s^{-1} from which the dissociation rate constant of the fast step was calculated to be $k_{-x} = K_x k_x = 0.35 \pm 0.05$ s^{-1}. Simulations of the time course of the reaction kinetics of the fast phase E + I* ⇌ X with these parameters showed good agreement with the experimental curves at all concentrations.

On the time scale of the slow phase X is in fast preequilibrium with E and I*. Therefore one has

$$[X] = \frac{K_x}{4}\left[\sqrt{1 + 4([E]_0 - [C])/K_x} - 1\right]^2 \tag{5}$$

The free enzyme concentration is calculated as

$$[E] = [E]_0 - [X] - [C]$$

$$= \frac{K_x}{2}\left[\sqrt{1 + 4([E]_0 - [C])/K_x} - 1\right] \tag{6}$$

Since k_{-c} is extremely small (see below), dissociation of C can be neglected and the rate equation reads

$$\frac{d[C]}{dt} = k_c[X] \tag{7}$$

The rate of decrease of free enzyme in the slow phase is obtained by differentiation of eq 6 and insertion of eq 7

$$\frac{d[E]}{dt} = -k_c \frac{[E]^2}{2[E] + K_x} \tag{8}$$

Inserting the initial condition $[C]_{t=0} = 0$ into eq 6 gives

$$[E]_{t=0} = \frac{K_x}{2}\left[\sqrt{1 + 4[E]_0/K_x} - 1\right] \tag{9}$$

Integration of eq 8 by separation of variables yields

$$k_c t = -2\left[\ln\frac{[E]}{[E]_{t=0}} - \left(\frac{K_x}{2[E]} - \frac{K_x}{2[E]_{t=0}}\right)\right] \tag{9a}$$

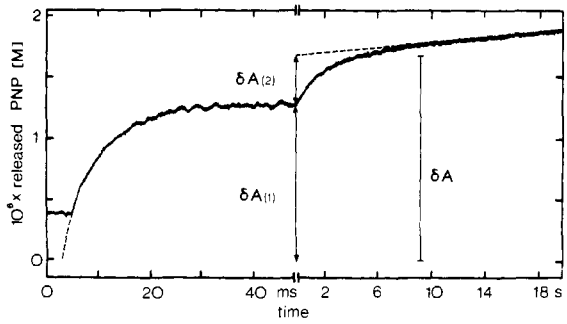

FIGURE 4: Addition of NPGB to a mixture of β-trypsin and I* at 82 s after mixing. $\delta A(1)$ and $\delta A(2)$ stand for the amplitudes of the two kinetic phases in which *p*-nitrophenol (PNP) is released and δA is the total amplitude. One syringe of the stopped-flow photometer contained a mixture of $[E]_0 = [I^*]_0 = 4 \times 10^{-6}$ M, the other $[NPGB]_0 = 5 \times 10^{-4}$ M. All solutions in 0.2 M triethanolamine buffer (pH 7.8) containing 50 mM $CaCl_2$ and 5.5% (v/v) DMF at $T = 22.5$ °C.

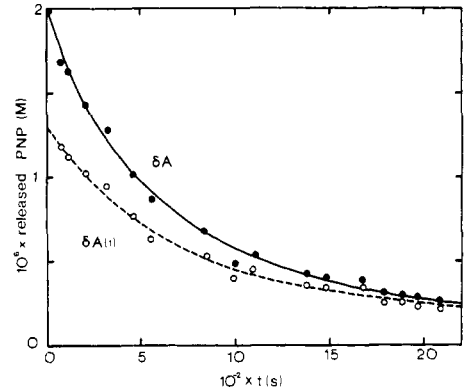

FIGURE 5: Time course of the amplitudes δA (●) and $\delta A(1)$ (○) expressed in released *p*-nitrophenol (PNP) after addition of NPGP. Conditions are as in Figure 4. The curves were calculated by numerical integration of eq 7 (—) and eq 8 (- - -), with $K_x = 4 \times 10^{-6}$ M and $k_c = 7 \times 10^{-3}$ s^{-1}.

At $t = T_{1/2}$ $[E] = [E]_{t=0}/2$ holds and eq 9a becomes

$$T_{1/2}(2) = \frac{2}{k_c}[\ln 2 + (1 + 4[E]_0/K_x)^{-1/2}] \qquad (9b)$$

This equation describes the experimental concentration dependence of $T_{1/2}$ (Figure 3) with $k_c = (1 \pm 0.1) \times 10^{-2}$ s^{-1}, and $K_x = (3.5 \pm 0.3) \times 10^{-6}$ M. With these values the time course of [E] in the slow phase was calculated by numerical integration of eq 8, since the analytical solution (eq 9a) cannot be resolved for the free enzyme concentration. Again a satisfying agreement with the experimental data was obtained at all concentrations. Also the time course observed by the intrinsic signal (Figure 2b) was quantitatively described by eq 8 using the values for k_c and K_x at pH 8.5 (see Figure 8) and assuming proportionality between the difference signal and the concentration of free enzyme.

Addition of NPGB during the Course of Association. In these experiments equimolar solutions of enzyme and modified inhibitor were mixed at time $t = 0$ and rapidly filled into one syringe of the stopped-flow apparatus. The other syringe contained the trypsin active site titrant NPGB in large excess. By mixing these two solutions at different times during the time course of the association of E + I*, it was possible to determine the amount of free trypsin, the concentration of X_1, and the rate constants k_{-x} and k_c.

A typical experiment is shown in Figure 4. The first phase of the kinetics is identified as the reaction of free trypsin with the active site titrant and its amplitude δA_1 is proportional to the amount of free trypsin [E] in eq 6. The second phase displays the dissociation of the intermediate X which dissociates after removal of free trypsin from the solution. Therefore the amplitude of this step δA_2 is proportional to [X] in eq 5 and the corresponding rate constant is k_{-x}. After completion of the reaction X → C no further dissociation was observed. This point was confirmed by following the reaction during several hours in a double beam photometer in which the slow spontaneous hydrolysis of NPGB could be compensated. The total amplitude $\delta A = \delta A_1 + \delta A_2$ is therefore proportional to $[E]_0 - [C]$. In Figure 5 δA and δA_1 are plotted vs. time t. The values of $K_x = (4 \pm 0.5) \times 10^{-6}$ M and $k_c = (6 \pm 0.5) \times 10^{-3}$ s^{-1}, which were obtained by a best fit of eq 7 and 8 to the data, were somewhat lower than those determined by proflavin displacement experiments. These differences are probably due to the presence of 5.5% DMF in the NPG experiments. The experiments reported in this section provide a direct demonstration of the dissociation of the intermediate X (Figure 4) and the amplitudes δA and δA_1 display the different time courses predicted by the differential eq 7 and 8 (see Figure 5).

Kinetic Control Dissociation during the Time Course of Association. These experiments were suggested by M. Laskowski, Jr. At time t after mixing of equimolar solutions of enzyme and modified inhibitor the reaction products were forced to dissociate by a sudden pH drop and the percentage of resynthesized virgin inhibitor I and of modified inhibitor I* was determined as described in the Methods section. The formation of I followed the kinetics of C formation as calculated by numerical integration of eq 7 using the parameters $K_x = 3.3 \times 10^{-6}$ M and $k_c = 1 \times 10^{-2}$ s^{-1}. This means that the complex formed from E and I* dissociates into inhibitor I and enzyme at pH 2.

Comparison of I Prepared in Different Ways and Resynthesis of the Reactive Peptide Bond.* The modified inhibitor I* used in these studies was prepared via several chemical steps. Although chemical evidence suggested that only the reactive site peptide bond in I was split by this procedure (Jering & Tschesche, 1974a,b, 1976a), it was desirable to compare the kinetic behavior of this I* preparation with that of I* prepared by direct enzymatic cleavage of I with plasmin at pH 5 (Tschesche & Kupfer, 1976). Another preparation was obtained by incubation of I with a protease from starfish at pH 10 (Estell et al., 1976). This material was a kind gift of Dr. M. Laskowski, Jr. The same type of association kinetics with β-trypsin was observed as described above (proflavin or NPGB method) with slightly lower values of K_x and k_c.

As another test of I* prepared according to Jering & Tschesche a small amount was reconverted to virgin inhibitor I by incubation with α-chymotrypsin for 70 days (Tschesche & Kupfer, 1976). In adcordance with the result of chemical and physical tests (Tschesche et al., 1974; Jering & Tschesche, 1976a,b), the kinetics of association of β-trypsin with this material was found to be indistinguishable from that of native virgin inhibitor.

Interaction of β-Trypsin with I-OMe.* Methylation of the Lys-15 carboxyl group in I* drastically changed the kinetic behavior. I*-OMe reacted with β-trypsin in a single irreversible second-order reaction as demonstrated by proflavin displacement and NPGB addition experiments. The reaction half-times as derived from proflavin displacement experiments at equimolar concentrations of enzyme and I*-OMe in 0.2 M triethanolamine buffer, pH 7.8, containing 50 mM $CaCl_2$ at $T = 22.5$ °C were found to be strictly proportional to $[E]_0^{-1}$. Eight values were measured in a concentration range of [I*-

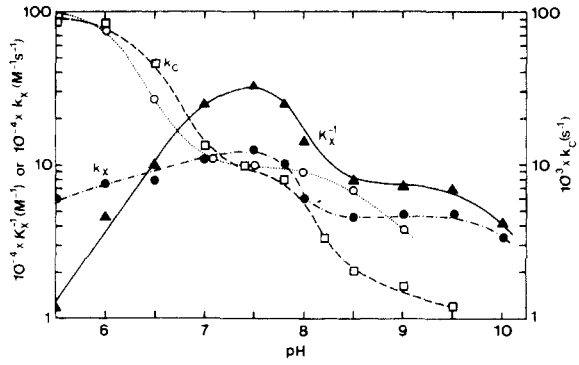

FIGURE 6: Dependence of the association of β-trypsin with I* on pH. Left ordinate: reciprocal preequilibrium constant K_x^{-1} (▲) and association constant k_x (●). Right ordinate: rate constant of X → C conversion k_c as determined by proflavin displacement (○) and by NPGB addition (□). The following buffers containing 0.2 M KCl and 0.05 M $CaCl_2$ were used: 0.05 M sodium acetate (pH 5–6), 0.05 M Tris–maleate (pH 6–7), 0.05 M Tris (pH 7.5–8.5), and 0.05 M sodium glycinate (pH 9–9.5), T = 22.5 °C.

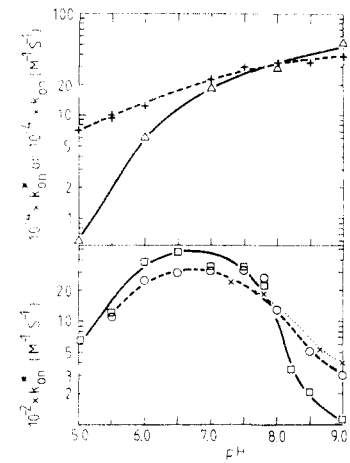

FIGURE 7: Comparison of the pH dependencies of the apparent rate constants k_{on}* for the association of β-trypsin with I* and I*-OMe. The data for I* (lower part of the graph) were calculated with eq 2a from the values of K_x and k_c shown in Figure 6 as determined by proflavin displacement (○), by NPGB addition (□), or by addition of substrate during the course of association (X). The data for I*-OMe (△) and virgin inhibitor (+) are shown in the upper part of the figure. Values were determined by the proflavin displacement method in the buffer system described in the legend of Figure 6.

OMe]$_0$ = [E]$_0$ = 4×10^{-7} to 6×10^{-6} M. The amplitude of the association reaction of I*-OMe with β-trypsin was also proportional to [E]$_0$ in the same concentration range which again indicates an essentially irreversible reaction with an equilibrium dissociation constant smaller than 5×10^{-8} M. The apparent rate constant of association k_{on}^* equaled $(2.2 \pm 0.4) \times 10^5$ M^{-1} s^{-1} at pH 7.5 which is only little lower than the corresponding k_{on} value of 2.9×10^5 M^{-1} s^{-1} for virgin inhibitor. When NPGB was added at different times after mixing enzyme and I*-OMe, no dissociation could be detected during 3 h. Kinetic control dissociation after six seconds of incubation of I*-OMe and β-trypsin [E]$_0$ = [I*-OMe]$_0$ = 2×10^{-5} M yielded 95 ± 5% virgin inhibitor.

Reaction of Des-(Ala^{16}, Arg^{17}) Inhibitor with β-Trypsin. The removal of the residues Ala-16 and Arg-17 from the active site of I* drastically increases the dissociation equilibrium constant of the reaction with trypsin (Jering & Tschesche, 1976a). Experiments with proflavin displacement and NPGB addition demonstrated a one step association with an equilibrium constant $K \geq 2.5 \times 10^{-4}$ M and an association rate constant of $k_+ = 5 \times 10^4$ M^{-1} s^{-1} at pH 7.5 in 0.05 M Tris buffer containing 0.2 M KCl and 0.05 M $CaCl_2$.

pH Dependence. The reaction of β-trypsin with I* was followed by the proflavin displacement method in the stopped-flow apparatus or the Cary 118 spectrophotometer ([E]$_0$ = [I*]$_0$ = 2×10^{-5} M, [proflavin]$_0$ = 5×10^{-5} M). The reaction was also monitored by the NPGB method ([E]$_0$ = [I*]$_0$ = 3.5×10^{-6} M, [NPGB]$_0$ = 2.5×10^{-4} M, 5.5% DMF).

The results are shown in Figures 6 and 7. In Figure 6 K_x^{-1} is plotted instead of K_x in order to emphasize the parallel pH dependence of K_x^{-1} and k_x at pH >7. One notes that the dissociation rate constant k_{-x} of the intermediate X is essentially independent of pH in this region. K_x^{-1} and k_x display a maximum at pH 7.5 and a broad shoulder at pH 8.5 to 9.5. In the pH range smaller than 6.5, K_x^{-1} decreases with a slope of −1. The rate constant k_c increases markedly with decreasing pH and has an inclination point at pH 7.5, where K_x^{-1} and k_x show maxima. Values determined by proflavin displacement and with NPGB differ in the low pH range perhaps due to the DMF in the NPGB experiments.

Figure 7 shows the pH dependence of k_{on}*. For I*, the pH profile shows a maximum, whereas with I*-OMe the dependence of log k_{on}* on pH exhibits a slope of −1 at pH <6 and levels off at pH >6. For comparison, the pH dependence of the association rate constant of virgin inhibitor with β-trypsin k_{on} is also shown. The latter is in agreement with the values previously determined by Finkenstadt et al. (1974).

Temperature Dependence. The temperature dependence of the association of β-trypsin with modified inhibitor was followed by proflavin displacement and by the NPGB method. The variation of K_x with temperature shows a small positive enthalpy change ΔH_x of 1.6 ± 0.5 kcal/mol. The activation energy of k_x was 10 ± 1 kcal/mol and that of k_c, 10 ± 2 kcal/mol.

Discussion

When virgin kallikrein inhibitor or virgin soybean inhibitor react with β-trypsin (Luthy et al., 1973; Finkenstadt et al., 1974; Quast et al., 1976) or with α-chymotrypsin (Quast et al., 1974), the formation of a Michaelis complex L precedes the formation of the final stable complex C. This first encounter is too fast to be resolved by stopped-flow techniques and it is probably a nearly diffusion-controlled reaction (Quast et al., 1974; Engel et al., 1974). The intermediate X which was demonstrated for the reaction of modified kallikrein inhibitor I* with β-trypsin in the present study is formed 50 to 1000 times more slowly and is about 100 times more stable than L. Therefore X is not the encounter complex. Most likely I* and enzyme first form a more labile complex L* at a diffusion-controlled rate. A complex L* with a similar stability as L was indeed observed for the reaction of modified soybean inhibitor with β-trypsin (Luthy et al., 1973). L* is therefore tentatively included in the following scheme which summarizes the rate constants at pH 7.5 and at room temperature:

$$E + I^* \rightleftharpoons L^* \underset{k_{-x} = 0.35\ s^{-1}}{\overset{k_x = 10^5\ M^{-1}\ s^{-1}}{\rightleftharpoons}} X \underset{k_{-2} = 3.5 \times 10^{-10}\ s^{-1}}{\overset{k_2 = 10^{-2}\ s^{-1}}{\rightleftharpoons}} C$$

$$C \underset{k_2\ 125\ s^{-1}}{\overset{k_{-2} = 8 \times 10^{-8}\ s^{-1}}{\rightleftharpoons}} L \underset{10^8 \leq k_1 \leq 5 \times 10^6\ M^{-1}\ s^{-1}}{\overset{4 \times 10^4 \leq k_{-1} \leq 2 \times 10^3\ s^{-1}}{\rightleftharpoons}} E + I$$

When the fast preequilibria are at the side of the free enzyme and inhibitor species, overall rate constants k_{on}*, k_x, and k_{on} are measured:

$$E + I^* \underset{k_{-x} = 0.35\ s^{-1}}{\overset{k_{on}^* = 3 \times 10^3\ M^{-1}\ s^{-1};\ k_x = 10^5\ M^{-1}\ s^{-1}}{\rightleftharpoons}} X \rightleftharpoons C \underset{k_{on} = 3 \times 10^5\ M^{-1}\ s^{-1}}{\overset{k_{-2} = 8 \times 10^{-8}\ s^{-1}}{\rightleftharpoons}} E + I$$

The intermediate X which is in fast preequilibrium with the free reactants E + I* converts to C with the slow rate constant of 10^{-2} s^{-1}. In the overall reaction, the stable complex C is reached about 100 times faster from the virgin side as reflected by the comparison of k_{on} with k_{on}*. Note that the L → C conversion is about 10 000 times faster than the X → C reaction. These differences are even larger for α-chymotrypsin (Quast et al., 1974; in preparation). The rate constant k_{-c} was calculated from k_c with the known equilibrium constants of the other steps and the equilibrium constant of hydrolysis K_{Hyd} = I*/I = 0.38 (Tschesche & Kupfer, 1977). This value is about 50 times larger than our earlier estimate (Quast et al., 1974). The value of $k_{-c} = 3.5 \times 10^{-10}$ s^{-1} corresponds to the enormous half-life time of about 100 years. Dissociation of C toward virgin inhibitor is about 20 times faster than dissociation into I* and E and k_{-c} cannot be determined experimentally.

Because of the high stability of C at neutral pH no dissociation of the final complex is induced by addition of an active site titrant. This served to discriminate between X and C since X dissociates completely into I* and E under the same conditions. Following rapid pH drops at various times of the reaction of E with I* again all the material which was complexed to X dissociated back to I* and E. The complex which had reacted further than X, however, dissociated quantitatively into virgin inhibitor and enzyme. This indicates that X converts directly into the final complex C and that no other stable intermediates between X and C are formed.

When the amino acid residues Ala-16 and Arg-17 were removed enzymatically from the active site of I* only weak complex formation with β-trypsin occurred at a rate which is comparable to the rate of formation of X. This demonstrates that the two residues in positions 16 and 17 and the possibility to resynthesize the peptide bond are of critical importance for the formation of the stabilizing contacts in the final complex C.

Some insight into the chemical nature of the intermediate X and its possible correspondence to the intermediates in catalytic proteolysis (Stroud, 1974; Blow, 1974) is obtained from the comparison of the kinetics of I* and I*-OMe. No slow step is observed in the reaction with I*-OMe. It can therefore be excluded that X → C is a step after the formation of the acyl complex. Any difference which may exist between the reactions of I* and I*-OMe should vanish after the *O*-methyl group is removed from Lys-15 in the acyl complex. The fact that I*-OMe is not only esterified in position Lys-15 does not really interfere with this argument since esterification of other carboxyl groups of the inhibitor has no influence on the association rate with trypsin. Furthermore the final inhibitor–chymotrypsin complexes dissociate with the same rate constant regardless whether they were prepared from I*-OMe, I, or I* (Quast et al., in preparation).

The absence of the slow X → C step in the case of I*-OMe and the pH dependence of k_c suggest that the carboxyl group of Lys-15 in I* and its state of ionization are of critical importance for the rate-determining reaction step in the X → C conversion. The rate constant k_c increases with decreasing pH by a factor of 100 in the pH range of 5.5 to 9.5. When only the fraction of protonated carboyxl groups would react, a maximum change by a factor of 10 per pH unit would be expected. The influence of other ionizable groups in the inhibitor and enzyme can, however, not be ignored since such effects are already exhibited in the fine structure of the pH profile of k_c. The large decrease of k_c with increasing pH leads to a steep decline of the pH profile of $k_{on}^* = K_x^{-1} k_c$ in the pH range of 7 to 9. Since K_x^{-1} also decreases with increasing pH in this range, the pH dependence of k_{on}* is even larger than that of k_c. By contrast there is only a small and opposite pH dependence of k_{on} for virgin inhibitor and of k_{on}* for I*-OMe in the same pH region. Since both I*-OMe and virgin inhibitor lack the carboxyl group at Lys-15, this observation supports the view that this group is responsible for the slow X → C conversion found with I*.

This interpretation is in agreement with the expectation that nucleophilic attack of the oxygen in the side chain of Ser-195 at the C-atom of the carboxyl group of Lys-15 is easy when this group is protonated or esterified but much more difficult or impossible in the case of the carboxylate group. This explanation would require that the X → C step with its characteristic pH dependence should be found for all inhibitor enzyme interactions of this type since the formation of the tetrahedral complex is a step in the general catalytic pathway for serine proteases. Indeed a very similar mechanism was found for the association of α-chymotrypsin with I* (Quast et al., in preparation). In the reaction of soybean trypsin inhibitor with β-trypsin, however, no slow step which can be compared with the X → C conversion was detected (Luthy et al., 1973). Therefore the possibility remains that the charged carboxyl group of Lys-15 in I* influences the kinetics via some unfavorable interactions with other unknown charged groups in the enzyme or inhibitor and that the X → C step cannot be related to any of the catalytic steps. It is unlikely that the slow step X → C with its pH dependence can be identified with the formation of the acyl complex from the first tetrahedral intermediate since such a step should be independent of pH. It is possible, however, that the difficult step is located even earlier in the mechanism. In any case we are lead to the conclusion that the important catalytic steps in the reaction of I* with the enzyme are all very fast as compared with earlier steps which imply the formation of a correct protein–protein contact and perhaps the formation of the first tetrahedral intermediate.

Although the pH dependencies of association rate constants are similar for I*-OMe and I in the alkaline range, the dependencies differ below pH 7. Only for I*-OMe a slope of 1 is reached for log k_{on}* vs. pH below pH 6.5. A similar dependence is found for ester hydrolysis catalysed by α-chymotrypsin (Hess, 1971) and was attributed to the protonation of the charge relay system of the enzyme. Although the p*K* of the charge relay system of 7 which was assumed in earlier work is presently disputed (Robillard & Shulman, 1974; Markley & Porubcan, 1976; Koeppe & Stroud, 1976; Krieger et al., 1976), the strong decrease of the catalytic function of serine proeases with decreasing pH is a well-established fact. For the reaction with virgin inhibitor the intactness of the charge relay system appears to be of less importance. This is suggested by the smaller pH dependence and is most directly demonstrated by the observation that a complex with similar stability and association rate constant is also formed with anhydrotrypsin (Vincent et al., 1974).

There are a large number of enzyme kinetic studies dealing with the hydrolysis of amide bonds in small substrates (for a review, see Hess, 1971) but little information exists on the mechanism of peptide bond resynthesis by serine proteases for other substrates than protein inhibitors. Yamashita et al. (1974) studied the resynthesis of peptide bonds in hydrolyzates of soybean globulin by chymotrypsin. In qualitative agreement with our finding these authors observed a bell-shaped pH profile for the rate of resynthesis. The rate decreased from pH 5 to 8 whereas for the corresponding proteolysis reaction an increase was observed in the same range. It was also found that the peptides esterified with methanol reacted much faster than the peptides with free carboxyl groups.

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