Kinetics of the Interaction of Bovine Pancreatic Trypsin Inhibitor (Kunitz) with α-Chymotrypsin†

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ABSTRACT: Stopped-flow studies on the association of α-chymotrypsin with pancreatic trypsin inhibitor were performed with the proflavine displacement method in a broad range of inhibitor concentration. When all corrections arising from the coupling of indicator binding were applied, good correspondence with other methods was observed. Results at neutral pH are in agreement with a mechanism in which a fast preequilibrium (diffusion-controlled association rate and dissociation equilibrium constant 5×10^{-4} M) is followed by a slow formation of the final complex (rate constant 350 sec^{-1}). The preequilibrium contributes about one-third of the total free energy of complex formation. The unfavorable positive enthalpy change connected with this step is overcompensated by a large positive entropy difference. The entropy contribution of the second step (precomplex to final complex) is also positive but in addition this step is favored by a small negative enthalpy. The pH dependence of the association rate is explained by the assumption that only α-chymotrypsin with unprotonated His-57 in its active site is reactive whereas the rate constant of the protonated species is at least three orders of magnitude smaller. The pH dependence of the dissociation rate is tentatively assigned to the protonation equilibrium of the shielded His-57 in the complex which has an unusual pK of less than 2.

Recently the structure of the complex between pancreatic trypsin inhibitor (PTI)[1] and trypsin has been elucidated by X-ray crystallography (Rühlmann *et al.*, 1973a,b). The PTI–α-chymotrypsin complex is very similar (Blow *et al.*, 1972). The structures of PTI (Huber *et al.*, 1970, 1971a; Deisenhofer and Steigemann, 1973) and of α-chymotrypsin (Birktoft and Blow, 1972) are also known.

From this wealth of structural information and from other physical and chemical information (for a review, see Laskowski and Sealock, 1971), it follows that PTI and an entire class of similar protein proteinase inhibitors may be looked at as "natural transition state analogs" of peptide substrates. A small section of the inhibitor chain (about seven residues long) resembles a substrate which is maintained by the folding of the rest of the chain in exactly the conformation needed for binding. In particular the bond angles of the carbon atom in the peptide carbonyl group to be attacked by the active serine of the protease (Ala-14–Lys-15 in PTI) are distorted from the normal values toward a tetrahedral configuration. This brings about large entropic and enthalpic advantages over a normal flexible substrate in which one special conformation as well as the tetrahedral configuration have to be formed by the interactions with the enzyme. Indeed binding constants of this class of inhibitors are unusually high (Laskowski and Sealock, 1971). The tetrahedral state was postulated much earlier as a high-energy intermediate in normal catalysis (Caplow, 1969; Fersht and Requena, 1971; see also Hess, 1971). It is found to be stable in the PTI–trypsin complex. There is little doubt that it also exists in PTI–α-chymotrypsin and very recently it was also demonstrated in the complex of soybean trypsin inhibitor (STI) with trypsin (Sweet *et al.*, 1973). The reaction steps which follow in normal catalysis (formation of an acyl intermediate and its hydrolysis) do not take place in the case of PTI and are very slow for other inhibitors leading to so-called modified inhibitors with one peptide bond split (Laskowski and Sealock, 1971). Rühlmann *et al.* (1973a,b) explain this by an immobilization of His-57 which is therefore prevented from delivering a proton to the peptide nitrogen and by the complete shielding of the contact region from solvent.

The finding that the binding of inhibitors of this class to serine proteases resembles the first step or steps of normal proteolysis together with the very detailed structural information summarized above makes kinetic investigations of the mechanism very attractive. In a pioneering work Luthy *et al.* (1973) demonstrated a two step mechanism for the binding of unmodified and modified STI to trypsin. Vincent and Lazdunski (1972, 1973), Schweitz *et al.* (1973), and Lazdunski *et al.* (1973) collected interesting kinetic data for a number of systems including PTI–α-chymotrypsin under conditions for which the reaction may be treated as a simple bimolecular binding process. In the present work it will be demonstrated that the two-step mechanism first postulated for STI–trypsin is also applicable to PTI–α-chymotrypsin. The latter system has the advantage that no conversion from unmodified to modified inhibitor has to be taken into consideration and that complications due to the pH-induced conformational change of trypsin (Finkenstadt *et al.*, 1973) are avoided.

Materials

Bovine pancreatic trypsin inhibitor (Kunitz) was a kind gift of Bayer AG, Leverkusen, Germany. Bovine α-chymotrypsin three-times crystallized was obtained from Worthington Biochemicals Corp., Freehold, N. J. Proflavine was purchased from Fluka, Buchs, Switzerland, and NPABC

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[1] Abbreviations used are: PTI, pancreatic trypsin inhibitor (Kunitz); STI, soybean trypsin inhibitor (Kunitz); NPABC, *p*-nitrophenyl N^2-acetyl-N^1-benzylcarbazate.

from Nutritional Biochemicals Corp., Cleveland, Ohio. All other chemicals were of reagent grade (Merck, Darmstadt, Germany, and Fluka).

If not otherwise stated, association rates and equilibrium constants were determined in the following buffer systems (final concentrations after mixing of the two solutions in stopped flow experiments are given): 0.1 M sodium phosphate in the pH range 4–8 and 0.05 M sodium glycinate + 0.05 M sodium phosphate + 0.05 M sodium chloride at pH 9 and 10. Dissociation rates were determined in 0.05 M sodium citrate buffer at pH 2.5 and 3 and in 0.05 M sodium acetate in the pH range 3.5–4.5. These two buffers contained 0.05 M sodium chloride. In the pH range 5–7, 0.1 M sodium phosphate buffer was used.

Methods

Association Rates. Measurements were performed with a stopped-flow spectrophotometer manufactured by Durrum Gibson, Palo Alto, Calif. The path length of the observation cell was 1 cm and the dead time of the instrument was determined to be 5 msec. The output signal $u[V]$ of the instrument is proportional to the transmittance T. As the change of transmittance $\Delta T \equiv T_\infty - T(t)$ associated with the reaction is only a small fraction of the transmittance T_∞ of the solution after the reaction, a known voltage u_∞ proportional to T_∞ is substracted by electrical compensation. The difference $\Delta u(t) = u_\infty - u(t)$ is recorded in a storage oscilloscope (type 549 Tektronix). Measurements are displayed in terms of normalized transmittance $\Delta T(t)/T_\infty = \Delta u(t)/u_\infty$ (see Figures 2 and 3). The change of absorbance ΔA is calculated from this quantity according to

$$\Delta A(t) \equiv A(t) - A_\infty = -\log (1 - \Delta T/T_\infty)$$

For the direct observation at 270 or 302 nm the syringes were filled with inhibitor and enzyme solutions of equal concentration (2×10^{-5} to 2×10^{-4} M). When the proflavine displacement method was used, one syringe contained the enzyme and the dye (typical concentrations 4×10^{-6} and 2×10^{-5} M, respectively) and the other was filled with the inhibitor solution. Most measurements were performed with inhibitor concentrations ranging from 2×10^{-5} to 6×10^{-4} M. The reaction was monitored at 465 nm at which wavelength the maximum difference signal $\Delta\epsilon = 1.75 \times 10^4$ M^{-1} cm^{-1} is observed.

All solutions were used within 2 hr after preparation. For measurements at pH >8, enzyme and proflavine were dissolved in 0.1 M sodium chloride solution at pH 6 and the buffer of the inhibitor solution was adjusted to a pH slightly higher than the pH desired to be measured after mixing. In this way the enzyme is exposed to the high pH only for the very short period in which the binding reaction takes place. This avoids complications arising from autolysis and from the much slower transition toward an inactive form of α-chymotrypsin (Fersht, 1972). All experimental values reported below are averages of at least seven measurements.

Dissociation Rates. At pH 2.5 and 3 the dissociation was followed in the Durrum-Gibson stopped-flow photometer at 465 nm. One syringe was filled with an equimolar mixture of inhibitor and chymotrypsin (about 4×10^{-5} M) in 0.1 M sodium chloride solution of pH 8.5, a pH at which complete complex formation takes place. The other syringe contained an about 8×10^{-5} M solution of proflavine in 0.1 M sodium citrate buffer of the desired pH.

In the pH range 3.5–5 the reaction was too slow for the Durrum-Gibson photometer. Therefore a simple self-made stopped-flow device was employed. This consists of two syringes which are emptied simultaneously by manual operation of a joint piston. The solutions mix in a T-shaped connection piece with an inner diameter of 0.15 mm. The mixture flows through a thin plastic tube into a commercial micro flow-through cell of 1-cm path length (Type 178, Hellma, Müllheim, Germany). The cell was placed into the sample compartment of a double-beam spectrophotometer (UV-200, Shimadzu, Kyoto, Japan), to which a recorder with chart speed 0.5 cm/sec was connected. The dead time of this setup was 0.5 sec. The reactant solutions were prepared as described above with the exception that the enzyme concentration was only 2×10^{-5} M. The reference cell was filled with the 1:1 mixture obtained after mixing. At pH 3 and 3.5 a prominent reaction phase of opposite amplitude is observed which is due to the dimerization of the enzyme (Quast *et al.*, in preparation) and which is so slow that it does not disturb the determination of the rate of complex dissociation. At pH 4, 4.5, and 5 the dimerization rate is comparable with the dissociation rate but complications were avoided by working at concentrations at which more than 85% of α-chymotrypsin is monomeric.

In the pH range 5–7 the α-chymotrypsin active-site titrant, NPABC (Elmore and Smyth, 1968), was used to effect an irreversible dissociation of the complex (De Vonis Bidlingmeyer *et al.*, 1972). This method is based on a photometric assay of *p*-nitrophenoxide N which is released in the reaction of NPABC with enzyme E. With free enzyme a very fast "burst" reaction is observed but for the reaction with α-chymotrypsin which is released from the complex C the dissociation rate constant k_{-2} is rate determining. It follows (subscript zero stands for total concentration and subscript s for sample cell)

$$[N]_s = [E]_0 - [C]_0 e^{-k_{-2}t} + \{[NPABC]_0 - ([E]_0 - [C]_0 e^{-k_{-2}t})\}(1 - e^{-k_H t}) \quad (1)$$

The last term accounts for the spontaneous hydrolysis of NPABC with a pseudo-first-order rate constant k_H. This effect is partially compensated when measurements are performed against a reference solution of the same concentration but without enzyme. In the reference cell *p*-nitrophenoxide with a molar absorptivity ϵ_N is released according to

$$[N]_R = [NPABC]_0(1 - e^{-k_H t}) \quad (2)$$

The change in absorption ΔA is given by

$$\Delta A = \epsilon_N([N]_s - [N]_R) = \epsilon_N([E]_0 - [C]_0 e^{-k_{-2}t})e^{-k_H t} \quad (3)$$

It is seen that full elimination of the disturbing effect caused by spontaneous hydrolysis is not achieved by the difference method employed by De Vonis Bidlingmeyer *et al.* (1972). Values of k_H were therefore determined and the measured kinetics was corrected according to eq 3. At pH 7 and 25°, $k_H = 2.7 \times 10^{-4}$ sec^{-1}. When the pH is lowered by one pH unit, k_H decreases by a factor of 10. Above pH 7, k_H becomes larger than k_{-2} and the method is not applicable.

Measurements were performed in a Shimadzu Model UV-200 double-beam spectrophotometer equipped with thermostatted cell holders. The wavelength was set depending on concentration and pH somewhere in the broad absorption region of *p*-nitrophenoxide. At pH 5 the absorption maximum is at 315 nm and at pH 7.5 it is near 400 nm. Wavelengths <320 nm were not used because NPABC absorbs in this region. Special cells were employed (Type 193 "for anaerobic operation," Hellma). These consist of the usual 1 × 1 cm optical compartment and a mixing chamber which is not in the op-

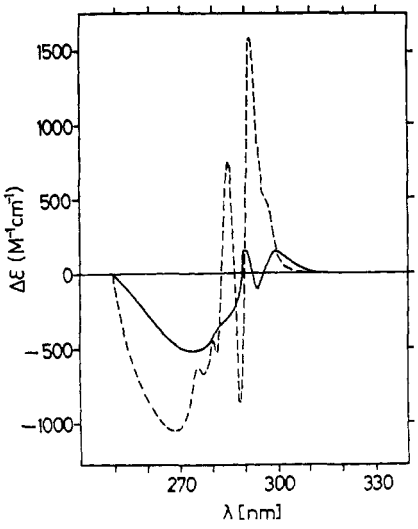


FIGURE 1: Difference in molar absorptivity between PTI–α-chymotrypsin complex and the free reactants. Tandem double cells (path length 0.43 + 0.43 cm) were filled as follows: sample cell, 2.75×10^{-5} M complex in compartment 1 and buffer in compartment 2; reference cell, 2.75×10^{-5} M α-chymotrypsin in compartment 1 and 2.75×10^{-5} M inhibitor in compartment 2. All in 0.1 M sodium phosphate buffer (pH 7). The difference spectrum of STI–trypsin (Luthy *et al.*, 1973) is shown for comparison (dashed curve).

tical path. The mixing chamber of the sample cell contained 0.500 ± 0.002 ml of 10^{-4} M complex solution and in some experiments α-chymotrypsin in excess. The mixing chamber of the reference cell was filled with the same volume of buffer. In a test tube 0.100 ml of a 4×10^{-2} M solution of NPABC in acetonitrile was mixed with 4.9 ml of buffer and the optical compartments of both cells were quickly filled with 2.000 ± 0.008 ml of this mixture. The cells were placed in the photometer and the base line was recorded for 3 min. Then both cells were removed and shaken simultaneously in order to mix the contents of the chambers with those of the cells. They were replaced and recording of the kinetics was started.

Some checks on the reliability of this method and in particular of the correction for hydrolysis were performed. It was found that results are not influenced by an excess of enzyme. Also with different complex concentrations identical results were obtained. At pH 5 a comparison with the proflavine method was possible and again good agreement was observed.

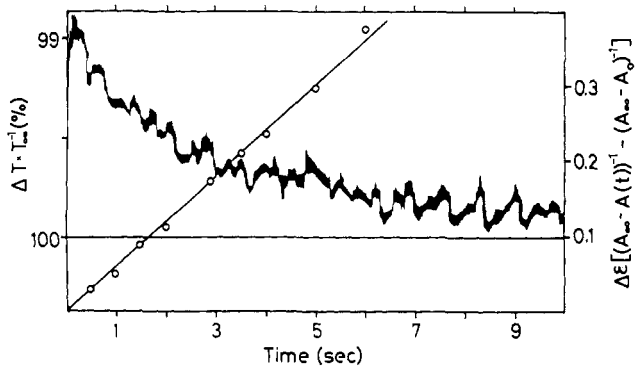


FIGURE 2: Oscilloscope trace of the normalized change in transmittance $\Delta T/T_\infty$ (see Methods) at 268 nm due to the intrinsic change of absorptivity which accompanies complex formation. The concentrations in the stopped-flow cell (10-mm path length) were 10^{-5} M for both enzyme and inhibitor in 0.1 M sodium phosphate buffer, pH 6 at 22.5°. The straight line represents a second-order plot of the same experiment (right ordinate).

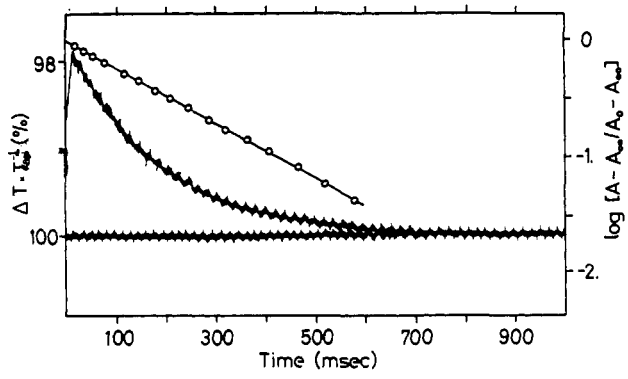


FIGURE 3: Oscilloscope trace of the normalized change in transmittance $\Delta T/T_\infty$ (see Methods) at 465 nm due to the displacement of proflavine by inhibitor. The concentrations in the stopped-flow cell (10-mm path length) were $[E]_0 = 2 \times 10^{-6}$ M, $[I]_0 = 2 \times 10^{-5}$ M, and $[P]_0 = 1 \times 10^{-5}$ M in 0.1 M sodium phosphate buffer, pH 8 at 14°. The straight line represents a first-order plot of the same experiment (right ordinate).

Diffusion coefficients were determined by the synthetic boundary method in an analytical ultracentrifuge (Model E, Beckman Instruments, Palo Alto). The inhibitor or the chymotrypsin were dissolved in 0.1 M sodium phosphate buffer (pH 6) and dialyzed against the same buffer for 2 hr. This time was sufficient for complete equilibration of salt concentration and short enough to prevent equilibration of the small inhibitor.

Results

Prominent Features of Stopped-Flow Kinetics. Signals in the difference spectrum are much smaller for PTI–α-chymotrypsin than in the case of STI–trypsin (Figure 1). A direct photometer display of the kinetics, which was successfully applied to the latter system (Luthy *et al.*, 1973), was therefore only possible in a very limited range of concentrations and at a 1:1 ratio of PTI and α-chymotrypsin ($[E]_0 = [I]_0$ from 10^{-5} to 4×10^{-5} M).

Measurements at low concentrations were performed at the wavelength of largest absolute signal (265–275 nm). At high concentrations the total optical density of the sample becomes limiting. Therefore a wavelength of 302 nm was chosen at which the relative signal $\Delta\epsilon/\epsilon = 0.014$ is larger than at 275 nm ($\Delta\epsilon/\epsilon = 0.008$). The results do not depend on wavelength. A typical oscilloscope trace is shown in Figure 2. The shape of the kinetic curves and the concentration dependence are usually consistent with a second-order reaction (Figure 2). A detailed and conclusive analysis is however hampered by the unfavorable signal to noise ratio and the small accessible concentration range. Kinetic constants derived from these traces are therefore mainly used to check the applicability of the more sensitive but also more complicated proflavine replacement method.

With the latter method it was possible to measure the association kinetics over a broad range of inhibitor concentration. Such constant enzyme and dye concentrations were chosen at which the replacement of proflavine by inhibitor causes a signal (at 465 nm) which is linearly related to the free enzyme concentration. With inhibitor in excess $[I]_0 \gg [E]_0$ (the index zero stands for "total concentration"), pseudo-first-order kinetics is expected. Indeed all oscilloscope traces collected at inhibitor concentrations higher than ten times the enzyme concentration can be perfectly described by a single relaxation time (Figure 3).

The signal to noise ratio is much better than in direct measurements and accurate values of the apparent first-order rate constants are obtained from logarithmic plots (Figure 3). When these rate constants are plotted *vs.* inhibitor concentration (Figure 4), a significant deviation from linearity is observed. The observed amplitude A also depends on inhibitor concentration. At $[I]_0 = 3 \times 10^{-4}$ M it has dropped to less than 70% of the value (A_0) obtained by extrapolation to $[I]_0 = 0$. Figure 4 suggests a proportionality between the reciprocal normalized amplitude $(A/A_0)^{-1}$ and $[I]_0$.

Formulation of a Mechanism. The simplest possible mechanism is the bimolecular reaction

$$E + I \underset{k_d}{\overset{k_a}{\rightleftharpoons}} C \qquad (4)$$

where E = enzyme, I = inhibitor, C = complex. Since the pseudo-first-order rate constant is $k_a[I]_0 + k_d$ for this mechanism, $t_{1/2}^{-1}$ ln 2 should be proportional to the inhibitor concentration. The deviation from linearity reported in Figure 4 clearly shows that the true mechanism must be more complex. It is useful however to define k_a as an *apparent* rate constant. Of course evaluation of k_a is only meaningful under conditions at which no, or at least no significant, deviation from second- or pseudo-first-order kinetics is observed. The most plausible extension of mechanism (4) is to postulate an intermediate complex L.

$$\begin{array}{ccccccc} & & P & & & & \\ & & + & & & & \\ E & + & I & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & L & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & C \\ {\scriptstyle k_p}\downarrow\uparrow{\scriptstyle k_{-p}} & & & & & & \\ J & & & & & & \end{array} \qquad (5)$$

We have chosen the same symbols as Luthy *et al.* (1973) who formulated the same mechanism for STI-trypsin. Since we are using proflavine (P) as an indicator in most experiments, the formation of a proflavine–enzyme complex (J) is also incorporated. The assumption made that P binds to E in a competitive way and not to I, L, or C will be discussed in the following paragraph.

Equilibrium constants are

$$K_L = k_{-1}/k_1 = [E][I]/[L]$$
$$K_C = k_{-2}/k_2 = [L]/[C] \qquad (6a\text{–}c)$$
$$Q = k_{-p}/k_p = [P][E]/[J]$$

Of course the overall dissociation constant of the complex is

$$K = K_L K_C \qquad (6d)$$

An intermediate L, if it exists, should influence the kinetic behavior but at equilibrium it should be present only in negligible concentration $[L] \ll [C]$, since a mixture of two different complex species has never been found, for example, by X-ray crystallography. This requires $K_C \ll 1$ or $k_{-2} \ll k_2$. These conditions are well met in the physiological pH range (pH >5), and although no difficulties in principle arise in the calculation of the general scheme it may usually be simplified by setting $k_{-2} = 0$.

For an interpretation of the kinetics it is important to know the origin of the signal by which the reaction is monitored. It is a shortcoming of the direct photometric method that it is not clear from *a priori* grounds which of the two steps (formation of L or L → C) is responsible for the difference signal. Luthy *et al.* (1973) concluded from kinetic arguments that the signal, or at least most of it, comes from the second step. With proflavine the observed change of absorption at 465 nm), ΔA, is proportional to the concentration of the enzyme–dye complex.

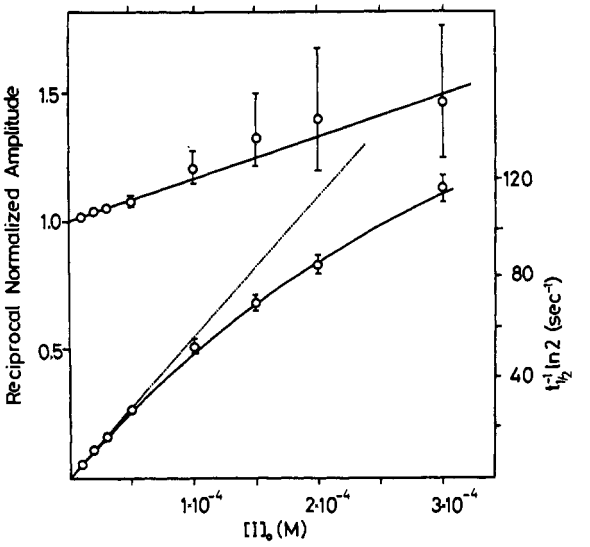


FIGURE 4: Dependence of the apparent first-order rate constant $t_{1/2}^{-1}$ ln 2 and of the reciprocal normalized amplitude on inhibitor concentration under pseudo-first-order conditions $[I]_0 \gg [E]_0 = 2 \times 10^{-6}$ M in 0.1 M sodium phosphate buffer, pH 8 at 22.5°. Proflavine concentration was 5×10^{-6} M. The circles represent the averages of at least seven independent measurements and the bars indicate calculated standard deviations. The curves were calculated from eq 11 and 12 with $K_L' = 5.9 \times 10^{-4}$ M and $k_2 = 350$ sec^{-1}. From the initial slope (dotted line) $k_{on}' = k_2/K_L'$ is obtained according to eq 14.

$$\Delta A = \Delta\epsilon[J] = \Delta\epsilon Q^{-1}([P]_0 - [J])([E] - [J]) \qquad (7)$$
$$[E] = [E]_0 - [L] - [C] \qquad (7a)$$

where $\Delta\epsilon$ is the difference between the molar absorptivities of J and P. [E] is the concentration of free enzyme which would be present in the absence of proflavine. It is desirable to measure [E] by a signal proportional to it. For this purpose we maintained $[E]_0 \ll Q$. It follows that $[J] \ll [P]_0$ can be omitted in the first bracket. On the other hand, $[J] \approx [E]$ because one has to work at $[P]_0 \approx Q$ in order to obtain sufficiently large signals. Under these conditions eq 7 reads

$$\Delta A/\Delta\epsilon = [J] = \{[P]_0/([P]_0 + Q)\}[E] \qquad (8)$$

The disadvantage of using this simple relation is that $[E]_0$ cannot be varied in a broad range without violating $[E]_0 \ll Q$. High concentrations of α-chymotrypsin may also cause complications due to the dimerization tendency of this enzyme (Aune and Timasheff, 1971). We therefore prefered to vary the inhibitor concentration only and performed most measurements under pseuso-first-order conditions $[I]_0 \gg [E]_0$. This procedure has the additional advantage that integration of the differential equations is easily possible.

For $k_{-2} = 0$ the rate equations are

$$d[L]/dt = k[E] - (k_{-1} + k_2)[L] \qquad (9a)$$
$$d[C]/dt = k_2[L] \qquad (9b)$$

with $k = k_1[I]_0$. Integration is performed with boundary conditions, which reflect the experimental conditions in stopped-flow experiments and the assumption $k_{-2} = 0$

$$\text{at } t = 0 \quad [L] = 0 \text{ and } [C] = 0$$
$$\text{at } t = \infty \quad [C] = [E]_0$$

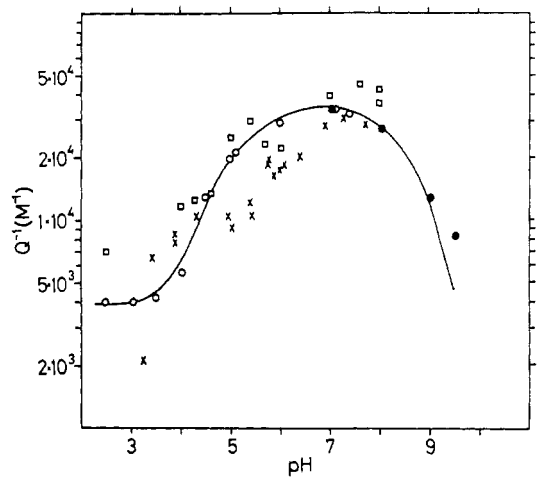


FIGURE 5: pH dependence of the binding constant Q^{-1} for proflavine–α-chymotrypsin. The curve was calculated for two acid–base equilibria in free chymotrypsin with pK = 5 and 8.7. The binding constants are: Q^{-1} (acid) = 4×10^8 M^{-1}, Q^{-1} (neutral) = 3.5×10^4 M^{-1}, Q^{-1} (basic) = 0. The equilibrium with pK = 8.7 represents the inactivation of α-chymotrypsin described by Fersht (1972). Our data (○) and data by Taylor *et al.* (1973) (●) are well represented by this curve. Data by Brandt *et al.* (1967) (□) and by Marini and Caplow (1971) (×) are also plotted.

and yields

$$[E] = \frac{k[E]_0}{\lambda_1 - \lambda_2}\left[\left(1 + \frac{k_2}{\lambda_2}\right)e^{\lambda_2 t} - \left(1 + \frac{k_2}{\lambda_1}\right)e^{\lambda_1 t}\right] \quad (10)$$

with

$$\lambda_{1,2} = -\frac{k + k_{-1} + k_2}{2} \pm \left[\left(\frac{k + k_{-1} + k_2}{2}\right)^2 - kk_2\right]^{1/2}$$

The time dependence of the disappearance of E which is measured by the change of A (eq 8) was evaluated from eq 10 for a number of special cases. Results are collected in Table I. It is found that if the formation of C *via* L may be regarded as a simple chain of consecutive reactions ($k_{-1} \ll k_2$) only the first step will show up. This has the entire amplitude (proportional to $[E]_0$) and has a rate constant $k = k_1[I]_0$ at all possible combinations of k and k_{-1}. At $k_{-1} = k_2$ (the intermediate case between consecutive reactions and fast preequilibrium), a slightly more complicated situation arises but again no saturation phenomenon like that shown in Figure 4 is predicted. Under all conditions where a single relaxation time is expected it is strictly proportional to $[I]_0$. A biphasic reaction which would follow for $k_{-1} \approx k_2 \approx k$ is not observed experimentally. We are therefore left with the case $k_{-1} \gg k_2$ which means that the reactants and L are in fast equilibrium compared with the formation of C. Independent of k and k_{-1} a very fast step and a slower phase well separated from it are predicted. The dependencies of the relaxation time and of the amplitude of the first step on $[I]_0$ are consistent with the results shown in Figure 4. The results in Table I have been calculated ignoring the presence of proflavine. For a quantitative comparison with experimental data corrections have to be applied which account for the fraction of enzyme occupied by the dye. For the apparent first-order rate constant of the slow step we find

$$\ln 2/t_{1/2} = [I]_0 k_2/([I]_0 + K_L') \quad (11)$$

and for its normalized amplitude

$$\frac{A}{A_{[I]_0 \to 0}} = 1 - \{[I]_0/([I]_0 + K_L')\} \quad (12)$$

K_L is obtained from the measured K_L' by

$$K_L = \{Q/([P]_0 + Q)\}K_L' \quad (13)$$

Equation 13 can be easily derived from eq 8 with $[E]_0 \ll Q$ and $[E]_0 \ll K_L$ because it is known that E + P ⇌ J constitutes another fast preequilibrium (see next paragraph) of L → C which competes with E + I ⇌ L. The validity of relation 13 has been experimentally checked at pH 6.5 by variation of the proflavine concentration $[P]_0$ from 3.8×10^{-6} to 3×10^{-5} M at constant $[E]_0 = 2 \times 10^{-6}$ M and constant $[I]_0 = 2.8 \times 10^{-5}$ M. A plot of K_L' *vs.* $[P]_0$ gave a straight line in accordance with eq 13. The intercept with the ordinate yielded K_L and from the slope the binding constant of proflavine Q (see next paragraph) was obtained. In addition, a plot of the amplitudes *vs.* $[P]_0$ gave the proper $\Delta\epsilon$ (see eq 8). The apparent rate constant k_a defined by eq 4 is now

$$k_a = k_2/([I]_0 + K_L) \quad (14a)$$

At low inhibitor concentrations $[I]_0 \ll K_L$, it follows that

$$k_a = k_2/K_L \equiv k_{on} \quad (14b)$$

In all cases in which a separate determination of K_L and k_2 is not possible, k_{on} is a useful quantity to be compared at different pH values. It is easily obtained from the initial slope of the graph $t_{1/2}^{-1} \ln 2$ *vs.* $[I]_0$.

Equilibrium and Kinetics of Proflavine Binding. For an application of eq 13 accurate values of Q are needed over the pH range of interest. In Figure 5 our data are plotted together with data taken from the literature. It has to be noted that the scattering of points may be due to different buffer systems. Our data and the data by Taylor *et al.* (1973) are well represented by the titration curve drawn for an acid–base pair with a pK = 5, Q^{-1} (protonated) = 3.6×10^3 M^{-1} and Q^{-1} (neutral) = 7×10^4 M^{-1}. In the basic region the theoretical curve was calculated according to Fersht (1972) who found a reversible inactivation (pK = 8.7) to an α-chymo-

TABLE I: Expected Time Dependence of $[E]/[E]_0$ for the Mechanism under Pseudo-first-order Conditions $[I]_0 \gg [E]_0$, $k = k_1[I]_0$.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} L \xrightarrow{k_2} C \qquad K_L = k_{-1}/k_1$$

	$k \gg k_{-1}$	$k \ll k_{-1}$	$k = k_{-1}$
Consecutive reactions $k_{-1} \ll k_2$	e^{-kt}		
Intermediate case $k_{-1} = k_2$	e^{-kt}	$e^{-kk_2t/(k_{-1}+k_2)}$	$0.27\,e^{-2.6kt} + 0.73\,e^{-0.38kt}$
Fast preequilibrium $k_{-1} \gg k_2$	$Xe^{-(k+k_{-1})t} + (1 - X)e^{-Xk_2t}$ with $X = [I]_0/([I]_0 + K_L)$		
	"fast"	"slow"	

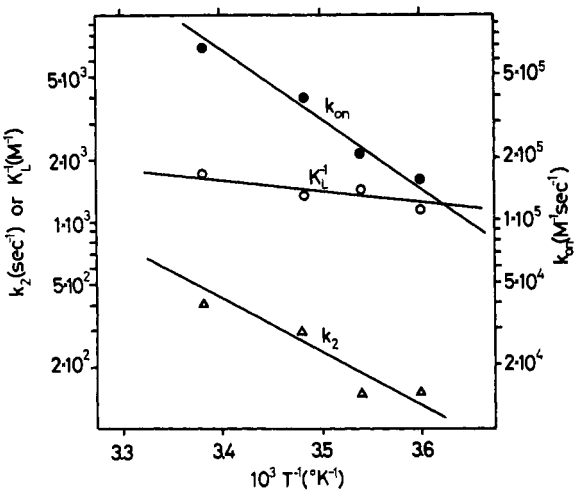


FIGURE 6: Logarithmic plot of rate constants k_{on} and k_2 and equilibrium constant K_L^{-1} *vs.* $1/T$. All measurements were performed in 0.1 M sodium phosphate buffer (pH 8). Activation energies are E_a = 13.4 kcal mol^{-1} for k_{on} and 10 kcal mol^{-1} for k_2. The enthalpy of the preequilibrium is 3.4 kcal mol^{-1}.

trypsin which does not bind proflavine. A 1:1 stoichiometry of the proflavine–enzyme complex was verified from Scatchard plots of the titration data (Heumann, 1972). Proflavine does not interact with PTI even at inhibitor concentrations as high as 10^{-3} M. This follows from the fact that no spectral changes of the dye are observed in the presence of PTI and from the successful application of the proflavine displacement method to evaluate the binding constant of PTI to α-chymotrypsin. Titration of chymotrypsin–proflavine complex with inhibitor (Heumann, 1972) gave proof for a 1:1 stoichiometry of both complexes and for the competitive nature of the displacement. Also the chymotryptic cleavage of *N*-acetyl-L-tyrosine ethyl ester is competitively inhibited by both PTI and proflavine (Heumann, 1972). It is difficult to show that proflavine does not bind to the intermediate complex L by a direct method. It was found however that identical values of K_L and k_a are obtained if correction 13 is applied to values of K_L' and k_a' measured at different concentrations of proflavine. Equation 13 would not hold true if binding to L occurs.

Concerning kinetics it is necessary to check that $k_{-p} \gg k_2$. By temperature-jump experiments the following values were obtained: at pH 5, $k_p = 1.2 \times 10^7$ M^{-1} sec^{-1} and $k_{-p} = 3.2 \times 10^3$ sec^{-1}; at pH 7, $k_p = 1.2 \times 10^8$ M^{-1} sec^{-1} and $k_{-p} = 8.3 \times 10^3$ sec^{-1}. These rate constants agree well with those reported for proflavine–trypsin by Guillain and Thusius (1970).

K_L and k_2. The experimentally observed dependence of the pseudo-first-order rate constant of association on inhibitor concentration (Figure 4) is well fitted by a curve drawn according to eq 11 with $K_L' = 6 \times 10^{-4} \pm 1 \times 10^{-4}$ M and $k_2 = 350 \pm 50$ sec^{-1}. After correction for the presence of proflavine (eq 13), we obtain $K_L = 5 \times 10^{-4}$ M. These values were evaluated at pH 8 and at 22.5°. The limits of error correspond to fits which are possible within the limits of standard deviations of the experimental points (bars in Figure 4). As an independent check, the linear dependence of the reciprocal amplitude expected for $K_L' = 6 \times 10^{-4}$ M was calculated from eq 12 and compared with experimental data. Although amplitude measurements are less accurate than those of half-life times, the agreement may be considered good.

By a similar procedure K_L and k_2 were determined at different temperatures. A Van't Hoff or Arrhenius plot of these data and of the temperature dependence of $k_{on} = k_2/K_L$ is shown in Figure 6. The thermodynamic parameters of the preequilibrium are calculated from K_L and from its temperature dependence. They are compared in Table II with the parameters of the overall complex formation, as obtained from K and from the difference between the activation energies of k_{on} and k_{-2}. The temperature dependence of k_{-2} (not shown) was measured in 0.1 M sodium phosphate buffer (pH 5). It yields an activation energy of 11 kcal mol^{-1}.

TABLE II: Standard Free Enthalpy $\Delta G°$, Standard Enthalpy $\Delta H°$, and Standard Entropy $\Delta S°$ at 22.5° in 0.1 M Sodium Phosphate Buffer at pH 8 for E + I → L and at pH 7 for E + I → C.

	$\Delta G°$ (kcal × mol^{-1})[a]	$\Delta H°$ (kcal × mol^{-1})[b]	$\Delta S°$ (eu)[c]
E + I → L	−4.4	+3.4	+26
E + I → C	−12.0	+2.4	+48
L → C[d]	−7.6	−1	+22

[a] $\Delta G° = RT \ln K_L$ and $RT \ln K$. [b] $\Delta H°$: for K_L from van't Hoff plot, for K from the difference between the activation energies of k_{on} and k_{-2}. [c] $\Delta S° = (\Delta H° - \Delta G°)/T$ at 22.5°. [d] The data for L → C are obtained from the difference between E + I → C and E + I → L.

Dependence of Equilibrium and Rate Constants on pH. For technical reasons it is very difficult to achieve unambiguous determinations of K_L and k_2 over a broad pH range in order to determine their pH dependence separately. It is however possible to determine k_{on} in the pH range 4–10 and to measure k_{-2} by a combination of various methods in the pH range 2.5–7. From these values the overall equilibrium constant K and its pH dependence are calculated. Results are summarized in Table III. This table also shows that reasonable agreement

TABLE III: pH Dependence of the Kinetic and Equilibrium Constants at 22.5°.

pH	k_{on} (M^{-1} sec^{-1})	k_{-2} (sec^{-1})	K (M)
2.5		6.0×10^{-1} [d]	
3.0		2.2×10^{-1} [d]	
3.5		4.7×10^{-2} [d]	
4.0	7.0×10^2 [a]	1.2×10^{-2} [d]	1.7×10^{-5} [f]
4.5	1.0×10^3 [a]	3.6×10^{-3} [d]	1.8×10^{-6} [f]
5.0	4.8×10^3 [a]	2.6×10^{-3} [d]	5.4×10^{-7} [f]
		2.0×10^{-3} [e]	8.3×10^{-7} [g]
5.5	1.5×10^4 [a]		
	6.7×10^4 [a]		
6.0	6.3×10^4 [b]	1.0×10^{-3} [e]	1.5×10^{-8} [f]
	6.5×10^4 [c]		
7.0	3.7×10^5 [a]	5.5×10^{-4} [e]	1.5×10^{-9} [f]
7.5	6.0×10^5 [a]		3.7×10^{-9} [g]
8.0	6.7×10^5 [a]		
	7.0×10^5 [c]		
8.8	5.5×10^5 [a]		
10.0	7.0×10^5 [a]		

[a] Proflavine displacement method with $[I]_0 \gg [E]_0$. [b] Direct monitoring by difference spectrum. [c] Proflavine displacement method with $[I]_0 = [E]_0$. [d] pH-jump monitored with proflavine. [e] With NPABC. [f] Calculated from $K = k_{-2}/k_{on}$. [g] Equilibrium titration with proflavine as indicator.

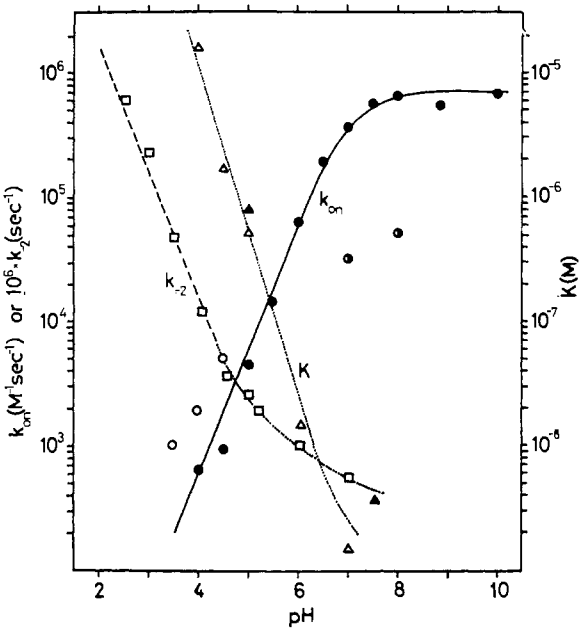
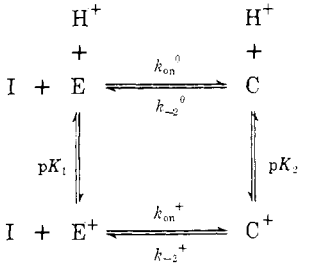


FIGURE 7: pH dependence of the apparent rate constant of association k_{on}, of the dissociation rate constant k_{-2} (left ordinate) and of the dissociation equilibrium constant K (right ordinate). Association rates were measured in sodium phosphate buffers (see Materials) (●); in 0.1 M sodium citrate buffer (○); in 0.1 M Tris buffer (◑). For buffer used for the determination of k_{-2} (□), see Materials. Values of K were either calculated from the kinetic constants (△) or determined by equilibrium titration: (▲) (Heumann, 1972). The drawn out curve is calculated for a single acid–base pair with pK = 7, k_{on}^0 (neutral) = 7×10^5 (M^{-1} sec^{-1}) and k_{on}^+ (protonated) = 0 (eq 16). For k_{-2} in the acid-pH range a slope of −1 is observed (dashed line). Experimental values which cannot be fitted by scheme 15 are connected by dotted curves.

exists between the data obtained by different methods. An attempt was made to fit the pH dependence of k_{on} (Figure 7) by the function expected for fast equilibrium between the unprotonated and the protonated species of the enzyme (E and E^+) and at $[I]_0 < K_L$



(15)

The apparent rate constant of association is now given by

$$k_{on} = \frac{1}{1 + 10^{pK_1 - pH}} (k_{on}^0 + k_{on}^+ \times 10^{pK_1 - pH}) \quad (16)$$

A reasonable fit to the experimental data is achieved with $k_{on}^0 = 7 \times 10^5$ M^{-1} sec^{-1} and pK_1 = 7. No clear indication for a leveling off is obtained at the lowest pH at which measurements were performed. This indicates that $k_{on}^+ < 10^3$ M^{-1} sec^{-1} and it was set equal to zero in eq 16 when the theoretical function in Figure 7 was calculated. At pH <6 the experimental pH dependence seems to have a slightly higher slope than d log k_{on}/dpH = 1 which would follow from eq 16 with $k_{on}^+ = 0$. It has to be taken into consideration, however, that rates do not only depend on pH but also on the concentration of buffer ions. Figure 7 shows that there are considerable differences when measurements are performed at pH 4.5 in sodium citrate or in sodium phosphate buffer. The largest difference exists between phosphate and Tris buffer. In the latter case k_{on} is smaller by a factor of 10 than in phosphate buffer (Figure 7). Even within a series of phosphate buffers of different pH values the concentration of ions varies and an ion effect may overlap the pH dependence.

The pH dependence of k_{-2} is in qualitative agreement with the reaction scheme 15 if it is assumed that the pK_2 of complex deprotonation is smaller than 2. This would explain the slope of −1 which extends down to pH 2. The rate constant of the fully protonated species k_{-2}^+ cannot be determined since the corresponding plateau is not reached. It may only be stated that $k_{-2}^+ > 1$ sec^{-1}. Also $k_{-2}^0 \approx 5 \times 10^{-4}$ sec^{-1} cannot be determined without unambiguity since no well-defined plateau is reached at neutral pH.

A serious contradiction to the simple scheme 15 arises when $K = k_{-2}/k_{on}$ is calculated from the k_{on} and k_{-2} values. From the scheme 15 the following pH dependence is derived

$$K = \frac{([E^0] + [E^+])[I]}{[C^0] + [C^+]} = K^0 \frac{1 + 10^{pK_1 - pH}}{1 + 10^{pK_2 - pH}} \quad (17)$$

According to this equation the slope d log K/dpH should never exceed −1. It is felt that the experimentally observed slope near to −2 at pH <6 can be only partially explained by the summation of experimental errors.

Discussion

The binding of PTI to α-chymotrypsin may be described by a simple bimolecular process only at very low reactant concentrations. This has been done by Vincent and Lazdunski (1973) who used extremely low concentrations in order to achieve reaction rates which were small enough for their slow kinetic methods. The kinetic data published by these authors (k_{on} and k_{-2} in our nomenclature) are mostly in rough agreement with our results. Their value of k_{on} at neutral pH 8 is 10 times smaller than our value and k_{-2} is about two times larger. A direct comparison is hampered by the use of different buffer systems. It was shown in the present study that ten times smaller k_{on} values are obtained in Tris buffer as compared with sodium phosphate buffer which was normally used by us.

Significant deviations from the kinetics of a bimolecular reaction which are observed at higher concentrations ($[I]_0 > 10^{-4}$ M) make it necessary to extend the mechanism. An intermediate complex is postulated and it is conclusively shown that this must be in fast equilibrium with the reactants. Such a mechanism which has been first proposed by Luthy *et al.* (1973) for STI–trypsin quantitatively explains all the kinetic results. At neutral pH all rate constants are determined or estimated to be as here given

$$[E] + [I] \underset{5 \times 10^4 > k_{-1} > 4 \times 10^3}{\overset{10^8 > k_1 > 8 \times 10^6}{\rightleftharpoons}} [L] \underset{k_{-2} = 6 \times 10^{-4}}{\overset{k_2 = 350}{\rightleftharpoons}} [C]$$

[k_1 (M^{-1} sec^{-1}) and all other constants (sec^{-1})]. The upper limit of k_1 is the maximum rate constants estimated for a diffusion-controlled reaction. The diffusion constants of PTI and α-chymotrypsin in 0.1 M sodium phosphate buffer (pH 6) at 22.5° are 15.8×10^{-7} and 10.5×10^{-7} cm^2 sec^{-1}, respectively. The radii of the molecules are obtained from models of their three-dimensional structures as 40 and 20 Å, respectively, and the "radii of the interacting sites" are estimated to be about 5 Å each. Applying Smoluchowski's (1917) formula in its modified form for the reaction of an active site on an enzyme with small ligands (Alberty and Hammes, 1958) a k_1

of 9×10^8 M^{-1} sec^{-1} is obtained. Since we are dealing with the reaction between the active sites of two large molecules, additional steric restriction arises and a value of 10^8 M^{-1} sec^{-1} appears to be a more realistic estimate.

A diffusion-controlled rate constant of 1.6×10^8 M^{-1} sec^{-1} was recently observed for the binding of phenylalanyl-tRNA to aminoacyl-tRNA synthetase (Krauss *et al.*, 1973). The molecular weight of the latter enzyme is about the same as that of chymotrypsin whereas the tRNA is about four times larger than PTI. The lower limit of k_{-1} follows from the fact that a fast preequilibrium is observed which is only possible if $k_{-1} \geq 10\,k_2$. The upper limit of k_{-1} and the lower limit of k_1 are obtained with the known equilibrium constant $K_L = 5 \times 10^{-4}$ M. The rate constants $k_1 = 6 \times 10^6$ M^{-1} sec^{-1} and $k_{-1} = 3 \times 10^3$ sec^{-1} which are reported by Hess *et al.* (1970) for the first complex of the substrate *N*-furylacryloyl-L-tryptophanamide with α-chymotrypsin, fall in the lower limits of this estimate. It is interesting to note that the equilibrium constants for the first complexes of the large aromatic substrates *N*-furylacryloyl-L-tryptophanamide (5×10^{-4} M) and *N*-furoylacryloyl-L-tyrosine ethyl ester (7×10^{-4} M) are about the same as K_L for PTI–α-chymotrypsin. The precomplex is less stable for smaller substrates like *N*-acetyl-L-phenylalanine ethyl ester (7×10^{-3} M) or *N*-acetyl-L-phenylalaninamide (3×10^{-2} M). A very weak complex is formed with the nonaromatic *N*-acetyl-L-leucine methyl ester (9×10^{-2} M). All data are taken from Hess (1971).

For PTI–α-chymotrypsin the preequilibrium contributes about one-third of the total free energy of complex formation. The enthalpy change connected with the formation of L is positive. This unfavorable contribution is overcompensated by the large gain in entropy. It is unlikely that entropy changes due to conformational transitions play a significant role in the process of complex formation. Almost identical structures have been found for both enzyme and inhibitor when they were investigated separately and in the complex (Rühlmann *et al.*, 1973a,b). In fact a small negative entropy change has to be expected for an association reaction in which phenomena like solvation, desolvation, or changes in solvent structure are unimportant. It is therefore felt that the positive entropy arises from changes in water structure and from changes of solvation. The first effect is assumed to be the main reason for hydrophobic interactions (for a review, see Nemethy, 1967).

The entropy of the second step L → C is also positive but in contrast to the first step the enthalpy change is slightly negative. This suggests that in the first complex L some specific interactions which contribute binding enthalpy in the final complex C are not yet formed because their formation is much slower than the dissociation of the precomplex. The latter is destroyed 10–100 times before one reaction step to C takes place. The process L → C is probably slow because of the very stringent sterical requirements which have to be met for a proper locking of the inhibitor binding region into the active site of the enzyme. Probably in the weaker precomplex, which is held together by not very orientation dependent hydrophobic interactions, inhibitor and enzyme can still assume a multiplicity of relative orientations. Even if these are in fast dynamic equilibrium the life time of the precomplex (2×10^{-4} to 2×10^{-3} sec) is not sufficient for the proper orientation for L → C to be found in more than 1 out of 10 to 100 instances.

The pH dependence of k_{on} follows the titration curve of a group with a pK of 7. It closely resembles the pH dependence observed for the rate of catalysis of α-chymotrypsin (Hess, 1971) and of methyl-α-chymotrypsin (Henderson *et al.*, 1972). The latter was explained by the assumption that the pK of 7 belongs to His-57 in the catalytic site of the enzyme which, if protonated, cannot participate in the charge relay system of the catalytic mechanism. By analogy we interpret our results by a mechanism in which only the fraction of enzyme with unprotonated His-57 can participate in complex formation, whereas the rate constant of the protonated species is at least three orders of magnitude smaller. This is in excellent agreement with the structural information that the inhibitor complex resembles the tetrahedral intermediate of enzyme catalysis, the formation of which is only possible if His-57 can accept the proton from Ser-195.

The rate constant of dissociation k_{-2} is smaller than k_2 by six orders of magnitude. For other protein inhibitor–enzyme systems even much smaller values have been measured (Laskowski and Sealock, 1971). Furthermore it was demonstrated (Lazdunski *et al.*, 1973) that, for systems of very different binding constants, k_{on} changes only by a factor of 10 whereas almost all the differences arise from different values of k_{-2}. This dissociation rate constant reflects the breakage of a number of relatively weak contacts which may differ in strength and number in different systems. It is the large number of contacts which have to be opened simultaneously, and not a special bond of particular strength, which make a dissociation process of this type very slow (Engel and Winkelmair, 1972).

The pH dependence of k_{-2} is not as easily interpretable as that of k_{on}. The slope $d \log k_{-2}/dpH = -1$, which is observed in the acid pH range, seems to indicate that again a single acid–base pair governs the dependence of pH. Only a lower limit can be estimated for the pK value (<2) since the plateau which has to be expected for the fully protonated species is not reached at the lowest pH at which measurements were possible. According to the simplest possible scheme 15 this pK should be that of His-57 in the complex. Such a pK shift of more than 5 would be the largest so far observed in proteins. On the other hand the shielding of His-57 from the exterior as a consequence of inhibitor binding (Rühlman *et al.*, 1973a) is perhaps the most extensive at present known in the structure of proteins.

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Characterization of an Endoribonuclease from *Xenopus* Oocytes. Possible Role in Ribonucleic Acid Turnover†

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ABSTRACT: On the basis of kinetic and base ratio data, a "4S" cytoplasmic RNA fraction may be an intermediate in the degradation of heterogeneous nuclear RNA (Aronson, A. (1972), *Nature (London), New Biol. 235*, 40). An endoribonuclease has therefore been purified and characterized from *Xenopus* oocytes. This enzyme preferentially hydrolyzes at pyrimidine residues splitting poly(U) 30 times faster than poly(C). There is no detectable hydrolysis of double-stranded poly(A)·poly(U) nor of DNA. The 3′ end of oligonucleotides produced by enzyme digestion are primarily uridine. The 3′ ends of "4S" RNA isolated from sea urchin embryo cytoplasm are also primarily uridine suggesting an *in vivo* function for this enzyme. The enzyme does not appear to be localized in the nucleus.

A very substantial fraction (85–94%) of the RNA synthesized by early sea urchin embryo turns over rapidly (Aronson and Wilt, 1969; Brandhorst and Humphreys, 1971). In fact, the time of synthesis of these molecules is very close to their half-life (A. I. Aronson, unpublished results). Most of the RNA synthesized by these embryos is rather large (about 3×10^6–10^7 daltons) with a base ratio close to that of the sea urchin DNA (Wilt *et al.*, 1969) and is classified as heterogeneous nuclear RNA (HnRNA). There is little if any detectable rRNA synthesis in these embryos prior to gastrulation (Emerson and Humphreys, 1970). Similar turnover of HnRNA in mammalian cells has also been described (Darnell *et al.*, 1973).

We had previously found a population of RNA molecules of approximately 4 S in the cytoplasm of hatched blastula embryos which on the basis of base ratio analysis and kinetic properties could have been an intermediate in the degradation of HnRNA (Aronson, 1972). The existence of this "4S" RNA suggested endonucleolytic cleavage of HnRNA so we began a search for an endonuclease which could be involved in turnover (Aronson, 1972). At the same time, we found an endonuclease in *Xenopus laevis* oocytes which appeared to

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