In this reaction, an intermediate not seen in the chymotrypsin-catalyzed hydrolysis of the specific substrate amides.

The $K'_{ns}$ values at pH 8.0 for the complexes of $\alpha$-chymotrypsin with N-acetyl-L-tryptophanamide and N-acetyl-L-tyrosylalaninamide were found to be 4.7 mM and 29 mM, respectively, in excellent agreement with the steady state kinetic parameter $K_{ns}$ (app). In the case of the $\alpha$-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester, data on the pH dependence of $k_{s}$ was found to be consistent with a pK (app) value of 6.6 for the group of the enzyme controlling the rate, and with a limiting pH-independent value for $k_{s}$ of 1800 sec$^{-1}$. The $K'_{s}$ value for this ester and $\alpha$-chymotrypsin at, for instance, pH 5.0, was found to be 2.3 mM, while $K_{ns}$ (app) has been reported as $\sim$0.08 mM. Values of $K'_{s}$ and $k_{s}$, determined in these experiments, together with previously determined values of the steady state kinetic parameter, $k_{cat}$, allowed a calculation of $K_{ns}$ (app). At pH 5.0, this calculated value is $\sim$0.06 mM, in reasonable agreement with the experimentally observed value.

Investigations of chymotrypsin-catalyzed reactions, although extensive, have relied almost exclusively on the steady state kinetic approach, and therefore very little is known about the individual steps in these reactions (1). We have undertaken an investigation of these steps in order to gain further understanding of the catalytic processes involved in the reactions.

The work was begun with a study of the reaction of chymotrypsin with its specific inhibitor diisopropyl fluorophosphate and with model substrates (2–8), because these reactions, in contrast to chymotrypsin-catalyzed hydrolyses of specific substrates, are known to proceed via long lived, covalently bonded chymotrypsin compounds (11–13). Physical changes in the enzyme which were observed to accompany these reactions were characterized; these include spectral changes near 290 m$\mu$ (2, 5), changes in optical
rotatory properties (4, 6, 14, 15), and changes in the acid dissociation constant of an ionizing group with a pK (app) of about 8.5 (4, 9, 10, 18, 17). Further information was obtained from kinetic investigations (8, 9) of the physical changes of the enzyme which accompany its stoichiometric reaction with diisopropyl fluorophosphate. We have now extended these investigations of individual reaction steps to the chymotrypsin-catalyzed hydrolysis of specific substrates, ester or amide derivatives of aromatic amino acids. In these reactions, only transient enzyme-substrate complexes are known to appear.

In an accompanying paper (18) we report preliminary kinetic as well as specific equilibrium investigations of the spectral changes of the enzyme near 290 μm which result when chymotrypsin binds the specific amide substrate N-acetyl-L-phenylalaninamide. These measurements (18) permitted the determination of an overall enzyme-substrate dissociation constant, K′′, and the pH dependence of this constant. Extension of the experimental method used in these studies to the chymotrypsin-catalyzed hydrolysis of specific substrates which are derivatives of tyrosine or tryptophan is, however, precluded by the high absorptivity of these residues in the 290 μm region. We have therefore used another approach to the determination of K′′ values and to the investigation of the pre-steady state kinetics of the chymotrypsin-catalyzed hydrolysis of specific substrates: a spectrophotometric procedure based on the competitive binding of the dye proflavin (3,6-diaminonaphthlene) by the enzyme.

The competitive inhibition of chymotrypsin by proflavin, first reported by Wallace, Kurtz, and Niemann (19), has been used by Weiner and Koehler (20) and Weiner et al. (21) as the basis of a determination of dissociation constants of complexes between chymotrypsin and "virtual" substrates by equilibrium dialysis techniques. This approach cannot be extended to real substrates because these compounds become hydrolyzed during the time required for equilibrium dialysis. In the present study, a spectrophotometric technique is used to measure, in the presence and absence of substrate, a perturbation of the absorption spectrum of proflavin which occurs upon binding to chymotrypsin. This perturbation, first reported by Bernhard and Lee (22) and Bernhard, Lee, and Tushjian (23), has been used to measure the dissociation constants for the proflavin complexes of both trypsin and chymotrypsin (24, 25), and for the detection of transient compounds in trypsin- and chymotrypsin-catalyzed reactions (23, 25). The magnitude of the difference spectrum, exhibiting a maximum at 465 μm, which appears when proflavin binds to chymotrypsin, is proportional to the amount of enzyme-proflavin complex. Also, the substrate itself forms a weak complex with proflavin which absorbs at 465 μm. Accordingly, the observed reduction of intensity of the difference spectrum depends on the relative concentrations of substrate and inhibitor, the dissociation constants of their complexes with the enzyme, the proflavin-substrate dissociation constant, and the molar absorbancy coefficients of the various proflavin complexes. Since the constants pertaining to the enzyme- and substrate-proflavin complexes can be determined independently, the chymotrypsin-substrate dissociation constant, K′′, can be calculated.

In the experiments reported in this paper, dissociation constants were determined at selected pH levels for the complexes of chymotrypsin with the amide and the ethyl ester of N-acetyl-L-tryptophan, and with the amides of N-acetyl-L-phenylalanine and N-acetyl-D-phenylalanine. We are also reporting the use of the proflavin-displacement method for determination of the pre-steady state kinetics in the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester over an extended pH range. This work includes the determination of rate constants not previously accessible experimentally.

In the experiments with specific amide substrates, static determinations could be made, since with amides the extent of hydrolysis during the time required for measurement is negligible. The experiments with the ester, however, required the use of stopped-flow techniques.

A preliminary report of a part of these data has appeared (26).

**EXPERIMENTAL PROCEDURE**

**Materials and Apparatus**

Salt-free α-chymotrypsin crystallized three times (Lots CDI 6148-9, CDI 6127-8, CDI 6164, and CDI 6094-5), trypsin crystallized twice, and crystalline chymotrypsinogen were obtained from Worthington. α-Chymotrypsin was prepared just before each use by activating chymotrypsinogen with trypsin under conditions known to yield essentially the α form of the enzyme (27). Enzyme concentrations were determined spectrophotometrically at 280 μm with use of a molar extinction coefficient of 50,000 M⁻¹ cm⁻¹ (98). A molecular weight of 25,000 (29) was assumed.

N-Acetyl-L-tryptophanamide, Lot R 4739, chromatographically pure, with melting point of 192–193° and [α]D +19.1 ± 1.2 (c, 1, in methanol); N-acetyl-L-phenylalaninamide, Lots K-4782 and K-46361, chromatographically pure, with melting point of 184–185° and [α]D +31° (c, 1.5, in methanol); and N-acetyl-D-phenylalaninamide, Lot K 3130, were obtained from Cyco Chemical Corporation. N-Acetyl-L-tryptophan ethyl ester, Lot J 1322, chromatographically pure, with melting point of 108–109°, and proflavin sulfate, Lot M 2200, with a molar extinction coefficient at 444 μm of 37,900 M⁻¹ cm⁻¹, were used from Mann. All other reagents were reagent grade and obtained from Mallinckrodt.

Cary recording spectrophotometers, either model 14 or model 15, equipped with both 0 to 1.0 and 0 to 0.1 slide wires, were used for measurements of difference spectra; 1.0- or 2.0-cm cells were used. Determinations of pH were made on a Radiometer pH meter, type TTT1c, with reference to Beckman pH 7.0 standard buffer. For the stopped flow experiments, a Gibson-Durrum stopped flow spectrophotometer was used. This instrument has a 2-cm light path and a tungsten iodide light source with grating monochromator.

**Methods**

**Determination of Enzyme-Substrate Dissociation Constants for Specific Substrate Amides—Difference spectra for enzyme-proflavin, substrate-proflavin, and enzyme-substrate-proflavin systems were measured in the 410 to 500 μm region at room temperature, 24° ± 0.3°. Since these proflavin-containing solutions showed maxima at 465 μm (22, 23), and since the absorption maximum for proflavin alone is at 444 μm, the difference in optical density readings between 465 μm and 440 μm, designated Δ[Δd]440-465, was used as a measure of the concentration of complex.** Data were handled as discussed under "Results."

Experimental solutions were prepared from stock solutions of...
Proflovin Concentration (pLM)

FIG. 1. Proflavin concentrations against optical density at 444 μm. Solutions, 0.1 M in potassium phosphate and 0.1 M in KCl at pH 8.0, were measured with a Cary model 15 spectrophotometer in 1.0-cm cells.

For the enzyme-proflavin systems, spectrophotometric traces were obtained for sample solutions containing enzyme and proflavin measured against reference solutions identical except that enzyme was omitted. Experiments at various pH levels were performed with sample solutions containing a constant concentration of proflavin and varying concentrations of enzyme, and in the case of α-chymotrypsin at pH 8.0 the experiment was also performed with solutions containing a constant concentration of enzyme and varying concentrations of proflavin (see Table I for solution compositions). Typical results are shown in Fig. 2.

For the proflavin-substrate systems, sample solutions containing a constant concentration of proflavin and varying concentrations of substrate (see Table II) were measured against reference solutions identical except for the absence of substrate.

For the enzyme-substrate-proflavin systems, positive difference spectra were obtained by making measurements with solutions containing enzyme and proflavin in the sample beam and solutions containing substrate in addition to the same concentrations of enzyme and proflavin in the reference beam. (For composition of the solutions see Table III). All measurements were completed within 3 to 4 min after addition of enzyme; during

\[ \Delta \text{OD} = \text{OD}_{444\mu m} - \text{OD}_{444\mu m} \]

The abbreviations used are: ATA, N-acetyl-l-tryptophanamide; APA, N-acetyl-l-phenylalaninamide; ATE, N-acetyl-l-tryptophan ethyl ester; n-APA, N-acetyl-n-phenylalaninamide.

### Footnotes
1. The abbreviations used are: ATA, N-acetyl-l-tryptophanamide; APA, N-acetyl-l-phenylalaninamide; ATE, N-acetyl-l-tryptophan ethyl ester; n-APA, N-acetyl-n-phenylalaninamide.

### Figures
- **Fig. 1**: Proflavin concentrations against optical density at 444 μm. Solutions, 0.1 M in potassium phosphate and 0.1 M in KCl at pH 8.0, were measured with a Cary model 15 spectrophotometer in 1.0-cm cells.
- **Fig. 2**: Tracing of Cary model 14 spectrophotometer recordings of difference spectra between α-chymotrypsin plus proflavin against proflavin. Measurements were made at pH 5.7 in solutions 0.1 M in potassium phosphate and 0.1 M in KCl. Cells used were 1.0 cm in size. Proflavin concentration was 4.0 μM. Concentrations of α-chymotrypsin: A, 163 μM; B, 122 μM; C, 102 μM; D, 81.6 μM; E, 61.2 μM; F, 40.8 μM.
this time only a negligible amount of substrate hydrolyzed, as shown by calculation from the known steady state kinetic parameters of these systems (31–33). In the most unfavorable cases in these experiments, the percentages of substrate hydrolyzed in 4 min, neglecting proflavin inhibition, are calculated to be 0% for 40 μM α-chymotrypsin and 1.0 mM ATA at pH 5.9, and 2% for 41 μM α-chymotrypsin and 2.0 mM APA at pH 8.0. The presence of proflavin reduces the amount of substrate hydrolysed by about one-third.

Determination of Enzyme-Substrate Dissociation Constants for Specific Substrate Amides and Investigations of Pre-steady State Kinetics—Experiments with ATP were performed in the stopped flow spectrophotometer at 28°C ± 0.3°C. Solutions containing α-chymotrypsin and proflavin in buffer were mixed according to the experimental stopped flow procedure with solutions containing substrate in the same buffer. Final concentrations were 10 μM α-chymotrypsin, 50 μM proflavin, and varying concentrations of substrate (see Table IV). For the experiments at pH levels 4.3, 4.6, 5.0, and 5.4, 0.1 M acetate buffer was used, and for the experiments at pH levels 5.7 and 6.0, 0.1 M phosphate buffer was used; all solutions contained KCl to give an ionic strength of 0.39. The solutions were brought to the desired pH by prior adjustment of the buffers with concentrated HCl. The time-dependent change in concentration of the enzyme-proflavin complex was then followed in the stopped flow apparatus at 465 nm.

Values of kobs were determined at the various substrate concentrations as discussed under "Results" (see Equation 19). Values of KES, the dissociation constant of the enzyme-proflavin complex, were determined at each pH value as described above in the method for determination of enzyme-substrate dissociation constants for specific substrate amides; these values are listed in Table I. From these data, and values of kobs obtained as kobs in steady state kinetic experiments, the constants k3 and K' s were determined by means of a digital computer program written for the Lineweaver-Burk form of the Michaelis-Menten rate equation (Equation 20 under "Results"). Data weighting and the calculation of the standard errors were performed as discussed by Wilkinson (34).

RESULTS

Determination of Dissociation Constants of Chymotrypsin-specific Substrate Amide Complexes—The calculation of K' s for a specific substrate amide depends on the following conditions and assumptions. (a) So >> Eo, Fo, so that S ≈ S0. Here E, S, and F refer to the molar concentrations of free enzyme, substrate, and proflavin, respectively, and the subscript zero indicates initial analytical concentration. (b) The equilibria between enzyme and substrate and between enzyme and proflavin are reached in a time that is short compared to the subsequent steps in which substrate is converted to product. Recent stopped flow experiments with the systems chymotrypsin + APA and chymotrypsin + APA + proflavin (18), in addition to the experiments reported here, indicate that this condition is fulfilled. (c) Substrate and proflavin are not bound simultaneously to the same enzyme molecule. Experiments at selected pH values indicate that chymotrypsin has only a single binding site for proflavin (20–24), and the experiments of Wallace et al. (19) and of Bernhard et al. (23) indicate that proflavin is a competitive inhibitor at pH 8.0 when acetyl-L-valine methyl ester or acetyl-L-tyrosine ethyl ester is the substrate. Then

\[
E_s = E + ES + EF_s
\]

and

\[
F_s = F + EF_s + SF
\]

where ES is the molar concentration of enzyme-substrate complex, EF s is the molar concentration of enzyme-proflavin complex in the presence of substrate, and SF is the substrate-proflavin complex. Also

\[
K_{EF} = \frac{(E)(F)}{EF}
\]

where EF is the molar concentration of enzyme-proflavin complex in the system, and

\[
K'S = \frac{(E)(S)}{ES}
\]

With appropriate use of Equations 1 and 2, Equation 3 becomes

\[
K' s = \frac{EF_s}{F} \left( \frac{K_{EF}(S_0)}{S_0} \right) \left( E_0 - EF_s \left( 1 + \frac{K_{EF}}{F} \right) \right)^{-1}
\]

Therefore, determination of EF s at various initial substrate concentrations allows computation of K' s according to Equation 4.

The required values of EF s can be calculated from difference spectrum data obtained by measuring solutions containing constant concentrations of enzyme and proflavin and varying concentrations of substrate against solutions identical except for absence of substrate. The pertinent equation is

\[
\Delta A_{465-400}^{obs} = \frac{(EF_s)(\Delta A'_{EF})}{(EF_s)(\Delta A'_{EF})} - \frac{(EF_s)(\Delta A'_{EF})}{(SF)(\Delta A'_{EF})}
\]

where ΔA'obs is the experimental difference spectrum measurement (see "Experimental Procedure"), EF represents the molar concentration of enzyme-proflavin complex in the absence of substrate, and EF s is, as above, the molar concentration of enzyme-proflavin complex in the presence of substrate. Needed for the calculation of EF s from this equation are the values of EF and SF, which can be calculated as

\[
EF = \frac{1}{2} \left( \frac{(K_{EF} + E_0 + F_0) \pm \sqrt{(K_{EF} + E_0 + F_0)^2 - 4E_0F_0}}{} \right)
\]

\[
SF = \frac{S_0 - K_{SF}}{E_0 - EF_s}
\]

Evaluation of Equations 6 and 7 requires knowledge of the dissociation constants for enzyme-proflavin, K_{EF}, and for substrate-proflavin, K_{SF}. In order to obtain these constants, separate experiments were performed with solutions containing either enzyme and proflavin or substrate and proflavin under conditions identical with those used in the experiments in which the solutions contained all three components. Calculation of these constants, K_{EF} and K_{SF}, from the experimental ΔA_{465-400}^{obs} values can be carried out according to Equations 8 to 10. In these equations, A represents molar concentration of either enzyme or substrate, C is molar concentration of the complex, K_{AP} represents either K_{EF} or K_{SF}, and the subscript zero indicates initial analytical concentration.

\[
K_{AP} = \frac{(n_0 A_0 C + O) (E_0 - O)}{C}
\]

where n represents the number of binding sites per molecule of A. When A0 >> C
and when \( F_0 \gg C \)

\[
\frac{1}{K_{AF}} = \frac{n}{A_0} \frac{\left( F_0 - C \right)}{\left( C - 1 \right)} = \frac{\Delta c_{AF}^{0}}{K_{AF}} \frac{\left( F_0 - n \Delta A_{AF}^{0} + n A_0 \right)}{\left( \Delta A_{AF}^{0} \right)^{2} + n A_0} \quad (9)
\]

and when \( F_0 \gg C \)

\[
\frac{1}{F_0} = \frac{1}{A_0} \frac{n A_0}{C - 1} = \frac{\Delta c_{AF}^{0}}{K_{AF}} \frac{n A_0}{\left( \Delta A_{AF}^{0} \right)^{2} + n A_0} - \frac{1}{K_{AF}} \quad (10)
\]

From Equations 9 and 10 it can be seen that a plot of \( A_0^{-1} \) or \( F_0^{-1} \) against \( \Delta A_{AF}^{0} \) in the manner of Benesi and Hildebrand (35) should give a straight line from which \( K_{AF} \) (which is either \( K_{EF} \) or \( K_{SF} \)) and \( \Delta M_{T} \) can be determined.

In the present work, the calculations of \( E_{F}, S_{P}, E_{F}, S_{P}, K_{EF}, \) and \( K_{SF} \) were carried out by computer, with use of \( K_{EF} \) values determined graphically. A program using Equations 4, 5, 6, 7, and 9 was written, and values of \( K_{SF} \) for each substrate concentration were obtained. In the calculations of \( K_{EF} \), a value of \( 2.6 \times 10^{4} \text{ M}^{-1} \text{ cm}^{-1} \) was used for \( \Delta A_{EF}^{0} \); this value was determined under conditions in which \( F_0 = 43 \mu \text{ M} \).

Table I includes the values of \( K_{EF} \) determined for the \( \alpha \)-chymotrypsin-proflavin complex at various levels of pH in the region 2.5 to 8.0, and the \( \delta \)-chymotrypsin-proflavin complex at pH 8.0. It can be seen in the table that these results are compatible with values obtained previously both spectrophotometrically (23, 24) and by equilibrium dialysis (20, 21). The agreement at pH 8.0 between the \( K_{EF} \) values obtained with constant enzyme and varied proflavin concentration, and with constant proflavin and varied enzyme concentration, indicates (see Equations 9 and 10) that only one proflavin binds per molecule of enzyme; this was found to be the case with \( \alpha \)-chymotrypsin and ribululbin also (36). The data in Table I also show that the binding of proflavin by \( \alpha \)-chymotrypsin decreases at low pH.

Table II lists the values obtained graphically for the dissociation constants, \( K_{EF} \), and molar extinction difference coefficients, \( \Delta A_{EF}^{0} \), of the complexes between proflavin and the various amide substrates.

The calculated values of \( K_{SF} \) for complexes between chymotrypsin and specific amide substrates ATA and APA are given in Table III, along with some comparative values of \( K_{EF} \) (app) obtained in other studies (33, 37, 38) of these substrates. It can be seen that reasonably good agreement was obtained between the statically determined \( K_{SF} \) and the kinetically determined \( K_{EF} \) (app) values. Also, a \( K_{SF} \) value for the \( \alpha \)-chymotrypsin-\( \beta \)-APA complex compares reasonably well with a \( K_{EF} \) determined from inhibition studies of the \( \alpha \)-chymotrypsin-catalyzed hydrolysis of chloroacetyl-L-valine methyl ester (39).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
Material & \( K_{EF} \) \\
\hline
\hline
\( \delta \)-Chymotrypsin (39 to 155 \mu M)-proflavin (4.0 \mu M), pH 8.0 & 4.9 \pm 0.2 \\
\hline
\( \alpha \)-Chymotrypsin (39 to 163 \mu M)-proflavin (4.0 \mu M), pH 8.0 & 2.7 \pm 0.1 \\
\hline
\( \alpha \)-Chymotrypsin (3.5 \mu M)-proflavin (10 to 60 \mu M), pH 8.0 & 2.3 \pm 0.4 \\
\hline
\( \alpha \)-Chymotrypsin (40 to 161 \mu M)-proflavin (4.0 \mu M), pH 8.0 & 4.4 \pm 0.4 \\
\hline
\( \alpha \)-Chymotrypsin (41 to 103 \mu M)-proflavin (4.0 \mu M), pH 5.7 & 4.3 \pm 0.4 \\
\hline
\( \alpha \)-Chymotrypsin (40 to 163 \mu M)-proflavin (4.0 \mu M), pH 5.0 & 4.3 \pm 0.4 \\
\hline
\( \alpha \)-Chymotrypsin (40 to 180 \mu M)-proflavin (4.0 \mu M), pH 4.0 & 4.3 \pm 0.4 \\
\hline
\hline
\hline
\end{tabular}
\end{table}

* \( K_{SF} \) determined by inhibition studies of the \( \alpha \)-chymotrypsin-catalyzed hydrolysis of chloroacetyl-L-valine methyl ester.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
System & \( K_{EF} \) & \( \Delta A_{EF}^{0} \) \\
\hline
\hline
ATA (2.5 to 14.0 \times 10^{-2} \text{ M})-proflavin (5.0 \times 10^{-4} \text{ M}), pH 8.0 & 3.2 & 3.6 \\
\hline
ATA (2.0 to 10.5 \times 10^{-2} \text{ M})-proflavin (4.0 \times 10^{-4} \text{ M}), pH 7.0 & 2.6 & 6.0 \\
\hline
ATA (1.0 to 7.1 \times 10^{-2} \text{ M})-proflavin (4.0 \times 10^{-4} \text{ M}), pH 2.5 & 4.5 & 8.3 \\
\hline
APA and D-APA (2.0 to 32.0 \times 10^{-2} \text{ M})-proflavin (4.0 \times 10^{-4} \text{ M}), pH 8.0 & 6.7 & 2.5 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
System & \( K_{EF} \) & \( \Delta A_{EF}^{0} \) \\
\hline
\hline
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\hline
ATA (2.0 to 10.5 \times 10^{-2} \text{ M})-proflavin (4.0 \times 10^{-4} \text{ M}), pH 7.0 & 2.6 & 6.0 \\
\hline
ATA (1.0 to 7.1 \times 10^{-2} \text{ M})-proflavin (4.0 \times 10^{-4} \text{ M}), pH 2.5 & 4.5 & 8.3 \\
\hline
APA and D-APA (2.0 to 32.0 \times 10^{-2} \text{ M})-proflavin (4.0 \times 10^{-4} \text{ M}), pH 8.0 & 6.7 & 2.5 \\
\hline
\end{tabular}
\end{table}

8.0 (32, 33) have indicated that \( \delta \)-chymotrypsin binds ATA about twice as well as \( \alpha \)-chymotrypsin. The accuracy of the static experiments reported here is not sufficient for a definite conclusion on this point to be drawn, but it does appear that the \( \delta \) form binds ATA better (Table III). In general, it can be concluded that the present results with static determination of \( K_{SF} \) for specific...
amidine substrates are in agreement with previous steady state kinetic measurements of $K_m$ (app).

**Stopped Flow Experiments with $ATE$ to Determine $K'_s$ and $k_{23}$**—As reported previously (26), the results of stopped flow experiments in which substrate is added to a mixture of enzyme and proflavin are different for specific substrate esters than for amides. Experiments in which $ATE$ is the substrate result in oscillo-

\[ R + S \xrightarrow{k_{12}} ES \xrightarrow{k_{23}} EP_2 \]

where $P_1$ is an alcohol or amine, $P_2$ a free acid, and $EP_2$ an acyl enzyme. In all the proflavin-displacement reactions, the first occurrence upon introduction of substrate (or buffer) appears to be a very rapid decrease in concentration of $EF$, the enzyme-proflavin complex. This decrease, which is just barely observable if seen at all (it does not appear in Fig. 3) is considered to reflect decomposition of $EF$ brought about by dilution or by formation of an enzyme-substrate complex, $ES$ (in Equation 11), or both. With the specific substrate amides $ATA$ and APA this initial rapid decrease in $EF$ concentration is followed by a time interval up to 5 mm in length, depending on $pH$ and $S_0$, during which $EF$ concentration remains constant, and which is considered to reflect the concentration of $ES$. It is this time interval that permits the static difference spectrum method of determining $K'_s$ described above for amides. With the specific substrate ester $ATE$, a second step observed in the proflavin-displacement reaction makes a direct measurement of $K'_s$ difficult. The determination of $K'_s$ for $ATE$ can, however, be made from the stopped flow measurements by the procedure outlined below.

The second step in proflavin-displacement reactions involving specific substrate esters (a step not observable in the case of the specific substrate amides) is a second decrease in $EF$ concentration (see Fig. 3), which is considered to occur as a result of the formation of another intermediate, such as $EP_2$ in Equation 11. This step, which has an observed rate dependent on both $pH$ and initial substrate concentration, is $S_0$ as shown in Fig. 3. This step is a half-time of 0.01 sec. Subsequent to this rapid but measurable decrease in $EF$ concentration, a period (about 5 sec in Fig. 3) is observed during which essentially no change in $EF$ occurs. The length of this period, which also depends on $S_0$, is considered to reflect the period during which there is maintained a steady state concentration of the intermediate such as $EP_2$. Finally, an increase in the concentration of $EF$ is observed; this is considered to be due to the decomposition of $EP_2$.

When stopped flow experiments such as the one illustrated in Fig. 3 are performed at various initial substrate concentrations, it is possible to calculate $K'_s$ and $k_{23}$ of Equation 11 under the following conditions or assumptions. (a) $ES$ and $EF$ are formed in a rapid pre-equilibrium phase of the reaction, and the subsequent progress of the reaction can be measured by observing the time-dependent change in $EF$ concentration. (b) $S_0 >> ES >> F_0$. These conditions allow the concentrations of both free substrate, $S$, and free proflavin, $F$, to be considered constant during the period of observation, and equal to their initial analytical values. Calculations indicate that under the most unfavorable experimental conditions used, the concentration of $F$ changes by not more than 8% during the development of the steady state. (c) $k_{12} >> k_{23}$. This assumption is warranted by the fact that the presence of a steady state intermediate cannot be detected in traces such as Fig. 3 and that the steady state kinetic parameter,

\[ R + S \xrightarrow{k_{12}} ES \xrightarrow{k_{23}} EP_2 \]

\[ k_{12} + k_{23} \]

Table III

<table>
<thead>
<tr>
<th>System</th>
<th>No. of measurements</th>
<th>$K'_s \times 10^3 M$</th>
<th>$K_m$(app)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-Chymotrypsin (4.0 $\times$ $10^{-4}$ m)-ATA (1.5 to 10.5 $\times$ $10^{-5}$ m), pH 8.0</td>
<td>12</td>
<td>4.7 $\pm$ 0.5</td>
<td></td>
</tr>
<tr>
<td>$\alpha$-Chymotrypsin-ATA, pH 7.9</td>
<td>19</td>
<td>6.3 $\pm$ 0.9</td>
<td>4.2 $\pm$ 0.4 (33)</td>
</tr>
<tr>
<td>$\alpha$-Chymotrypsin-ATA, pH 5.7</td>
<td>18</td>
<td>14.4 $\pm$ 4.5</td>
<td>3.3 $\pm$ 0.3 (39)</td>
</tr>
<tr>
<td>$\alpha$-Chymotrypsin-ATA, pH 6.0</td>
<td>18</td>
<td>3.9 $\pm$ 1.5</td>
<td>5.4 $\pm$ 0.9 (33)</td>
</tr>
<tr>
<td>$\alpha$-Chymotrypsin-ATA, pH 2.5</td>
<td>18</td>
<td>1.8 $\pm$ 0.1 (33)</td>
<td></td>
</tr>
<tr>
<td>$\alpha$-Chymotrypsin (1.0 $\times$ $10^{-3}$ m)-ATA (1.0 to 7.1 $\times$ $10^{-3}$ m), pH 2.5</td>
<td>18</td>
<td>28 $\pm$ 7</td>
<td>31 (37)</td>
</tr>
<tr>
<td>$\delta$-Chymotrypsin (2.0 $\times$ $10^{-4}$ and 4.0 $\times$ $10^{-4}$ m)-APA (2.0 to 21.2 $\times$ $10^{-5}$ m), pH 8.0</td>
<td>13</td>
<td>6.6 $\pm$ 0.5</td>
<td>12 $\pm$ 3$^b$ (38)</td>
</tr>
</tbody>
</table>

The diffusion constants, $K'$, for chymotrypsin-specific amide substrate complexes

Results obtained by the proflavin-displacement method under steady state conditions are compared to $K_m$ (app) values determined previously. Temperature was 24°C. The concentration of proflavin was $4.0 \times 10^{-5}$ M throughout the proflavin displacement experiments.

TABLE III

Dissociation constants, $K'_s$, for chymotrypsin-specific amide substrate complexes

As reported previously (26), the results of stopped flow experiments in which substrate is added to a mixture of enzyme and proflavin are different for specific substrate esters than for amides. Experiments in which $ATE$ is the substrate result in oscil-
Determination of $k_{at}$ and $K'_{s}$ for complex of $\alpha$-chymotrypsin and ATE from stopped flow experiments

Measurements were made spectrophotometrically at 465 nm by the proflavin-displacement method. Concentrations of enzyme and proflavin in the reaction mixtures are approximately 10 $\mu$M and 50 $\mu$M, respectively; ionic strength is 0.39; temperature was 28°.

<table>
<thead>
<tr>
<th>pH</th>
<th>ATE concentration</th>
<th>$k_{at}$</th>
<th>$K'_{s}$</th>
<th>$k_{at}$</th>
<th>$K_{m} (app)$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$M$</td>
<td>$sec^{-1}$</td>
<td>$M \times 10^{6}$</td>
<td>$sec^{-1}$</td>
<td>$M \times 10^{6}$</td>
</tr>
<tr>
<td>6.0</td>
<td>0.5-2.7</td>
<td>138 ± 10</td>
<td>0.6 ± 0.1</td>
<td>3.0 (37)</td>
<td>8.7 (37)</td>
</tr>
<tr>
<td>5.7</td>
<td>0.5-2.5</td>
<td>156 ± 26</td>
<td>1.7 ± 0.3</td>
<td>2.0 ± 0.3</td>
<td>3.3</td>
</tr>
<tr>
<td>5.4</td>
<td>0.5-2.7</td>
<td>103 ± 13</td>
<td>2.3 ± 0.5</td>
<td>0.84 (37)</td>
<td>8.3 (37)</td>
</tr>
<tr>
<td>5.0</td>
<td>0.5-2.25</td>
<td>35 ± 9</td>
<td>2.9 ± 0.2</td>
<td>0.35 (37)</td>
<td>6.6 (37)</td>
</tr>
<tr>
<td>4.6</td>
<td>0.6-2.75</td>
<td>17.5 ± 1.3</td>
<td>3.0 ± 0.2</td>
<td>8.7 (37)</td>
<td></td>
</tr>
<tr>
<td>4.3</td>
<td>0.6-2.75</td>
<td>8.2 ± 1.2</td>
<td>5.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The experimentally observed values $K'_{s} (1 + (F_{o}/K_{BF}))$ (see Equation 20) were converted to $K'_{s}$ by assuming an average value for $K_{BF}$ of $4.4 \times 10^{6} M$ at all pH values.

$k_{at}$ is small compared to $k_{obs}$ in Equation 16c. The steady state kinetic constant $k_{at}$ can therefore be considered a measure of $k_{at}$.

(d) Proflavin is a competitive inhibitor for the substrate and does not perturb the system in some unknown way.

The pertinent equations are as follows. In these equations, $E, F, S, P, EF, SF$, and $EP_{2}$ refer to the molar concentrations of these substances, and the subscript zero indicates initial analytical concentration (total concentration of the substance in all its forms).

$$K_{EF} = \frac{(E)(F_{o})}{EF}$$

or

$$K_{EF} = \frac{E}{F_{o}}$$

$$E = \frac{K_{EF}}{K'_{s}}$$

$$ES = \frac{(E)(S_{o})}{K'_{s}} = EF \frac{K_{EF}S_{o}}{F_{o}K'_{s}} = EP_{3}$$

$$EP_{2} = E_{o} - EF \left( \frac{K_{EF}}{F_{o}} + \frac{K_{EF}S_{o}}{F_{o}K'_{s}} + 1 \right) = E_{o} - EP_{3}$$

$$\frac{d(EP_{2})}{dt} = k_{o}S - k_{at}EP_{3}$$

Integrate Equation 15 gives the concentration of $EF$ at time $t$.

$$\frac{d(E_{o} - EP_{2})}{dt} = EF (k_{at}EP_{3} + k_{at}P_{3}) - k_{at}EP_{3}$$

FIG. 3. Photographs of oscilloscope traces of transmittance at 465 nm in a stopped flow experiment with ATE at pH 6.0 (see Table IV). The time scale for the first part of the experiment is 0.02 sec per cm; for the other part shown it is 5.0 sec per cm. Recording of the initial fast increase in transmittance, not shown, would have required a third time scale. Temperature was 28°.

FIG. 4. Stopped flow data at 465 nm for ATE added to a mixture of $\alpha$-chymotrypsin and proflavin. A function of absorbance at time $t$ and at steady state is plotted against time in accordance with Equation 19 of the text; the slopes of the experimental lines yield $k_{obs}$. Final solution concentrations are 10 $\mu$M $\alpha$-chymotrypsin and 50 $\mu$M proflavin; ionic strength = 0.39; temperature = 28°. $\bigcirc$, pH 5.4, initial concentration of ATE solution ($S_{o}$) = 2.0 $\mu$M; $k_{obs}$ = 38.7 sec$^{-1}$. $\bullet$, pH 5.4, $S_{o}$ = 0.90 $\mu$M; $k_{obs}$ = 19.9 sec$^{-1}$. $\triangle$, pH 4.6, $S_{o}$ = 2.75 $\mu$M; $k_{obs}$ = 5.7 sec$^{-1}$. 

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is the molar absorption coefficient of the enzyme-proflavin complex at 465 μm. Therefore, a plot according to

\[
\ln [(A_{466})_t - (A_{465})_s] = C - k_{obs} t \tag{19}
\]

allows evaluation of \(k_{obs}\). Typical examples of first order plots of the pre-steady state portion of the stopped flow traces according to Equation 19 are shown in Fig. 4. The dependence of \(k_{obs}\) on the scope, on substrate concentration is seen in the two plots at pH 5.4. The effect of pH on \(k_{obs}\) is shown by the two plots at the same substrate concentration but different pH.

Values of \(k_{obs}\) obtained at various substrate concentrations can then be plotted according to a rearrangement of Equation 16a

\[
k_{obs} = k_{32} - \left[ \frac{k_{obs}}{S_0} K'_S \left( 1 + \frac{S_0}{K_{EF}} \right) \right] \tag{20}
\]

Since \(S_0\), \(K_{EF}\), and \(k_{32}\) are known, \(k_{32}\) and \(K'_S\) can be determined either graphically or, as in this study, by means of a digital computer program.

Fig. 5 shows typical plots of the data according to Equation 20. The lines, for experiments at pH 5.0 and pH 5.4, are those calculated with the computer program, but are presented in the form of an Eadie plot (42).

\[
\text{Fig. 5. Values of } k_{obs} - k_{32} \text{ determined by stopped flow experiments with ATE plotted against } (k_{obs} - k_{32})/S_0 \text{ where } S_0 \text{ is initial ATE concentration. In accordance with Equation 20 of the text, } k_{32} \text{ and } K'_S \text{ can be obtained from these plots (see Table IV). } \bigcirc, \text{pH 5.0. } \bullet, \text{pH 5.4.}
\]

The steady state concentration of EF is obtained by use of Equations 12 and 13 and the relation \((EF)_s = k_{32} ES/k_{34}\). Substitution into Equation 14 and rearrangement gives

\[
E_0 - (EF)_s = k_{32} S_0 \left[ \frac{k_{32} S_0}{k_{34} + k_{33}} + k_{34} \right] \tag{17}
\]

Therefore

\[
(EF)_s = \frac{k_{32} S_0 \phi_3}{k_{32} \theta_1 + k_{34} \phi_2} \tag{16a}
\]

Then

\[
(EF)_t - (EF)_s = \frac{1}{\varepsilon_{EF}} [(A_{466})_t - (A_{465})_s] \tag{18}
\]

\[
= \frac{k_{32} \phi_3}{k_{32} \theta_1 + k_{34} \phi_2} (e^{-k_{obs} t})
\]

where \(A_{465}\) is the absorbance of the solution at 465 μm and \(\varepsilon_{EF}\)

\[
\text{Fig. 6. Observed values of } k_{32} \text{ for the ATE stopped flow reaction plotted as a function of hydrogen ion concentration. These data are compared with a line drawn according to the relation}
\]

\[
\frac{1}{k_{32}} = \left( \frac{1}{k_{32} \text{ (lim)}} \right) (H^+)^{0.6} + \frac{1}{k_{32} \text{ (lim)}}
\]

which is a rearrangement of Equation 21 in the text. These calculations for the line were made with use of a \(k_{32} \text{ (lim)}\) value of 1800 sec⁻¹ and a \(K'_e\) value corresponding to a contouring group of pH (app) = 6.8.

\[
\frac{1}{(A_{465})_t} - \frac{1}{(A_{465})_s} = C - k_{obs} t
\]
In Table IV are tabulated the values and standard deviations obtained for $K_s'$ and $k_{23}$ for ATE in the pH region 4.3 to 6.0. Also shown for comparison are previously published (37) steady state kinetic data, and values of $K_m$ (app) calculated from experimentally determined constants in accordance with the relation $K_m$ (app) = $K_m' g(k_{23} + k_{34})$ (41). It can be seen that observed and calculated $K_m$ (app) values are in reasonable agreement considering that the calculated values are based on determinations of so many constants.

It also appears from the data in Table IV that $k_{23}$ is pH dependent, although the accuracy of the data and the limited pH range investigated (above pH 6 the reactions are too fast to be measured by our stopped flow equipment) are not sufficient for definite conclusions to be drawn. In Fig. 6 the data in Table IV are plotted according to a rearrangement of the equation

$$k_{23} = k_{23} (\text{lim}) \left( \frac{K_s'}{K_s' + [H^+]} \right)$$

where $k_{23}$ is the observed rate at a given pH, $K_s'$ is the observed acid dissociation constant of an ionizing group of the enzyme which controls the rate, and $k_{23} (\text{lim})$ is the limiting, pH-independent value of $k_{23}$ reached at the pH at which the ionizing group is completely dissociated. Fig. 6, in which $k_{23}^{-1}$ is plotted against $[H^+]^n$, shows that the $k_{23}$ values obtained in the pH 4.3 to pH 5.7 region (Table IV) are consistent with but do not determine a pH-independent value for $k_{23}$ of 1800 set? and a reaction revealed that these data also fail to give evidence for the existence of an enzyme-substrate complex such as $ES$ in Equation 11. Recent investigations by Barman and Gutfreund (46) of the chymotrypsin-catalyzed hydrolysis of furylacryloyl-L-tyrosine ethyl ester suggested to these authors the existence of an additional intermediate between $ES$ and $EP_3$ in Equation 11. In this work, however, the only experiment reported with this substrate was performed under conditions such that initial enzyme concentration was approximately the same as initial substrate concentration, and quantitative interpretation of the data under these conditions requires the evaluation of non-linear differential equations, an operation the authors (46) did not attempt.

Interpretation of the data we have obtained so far is also hampered by some difficulties. (a) Due to limited solubility of ATE, it was not possible to use initial substrate concentrations that were sufficiently greater than $K_s'$ to make the values of $k_{23}$ and $K_s'$ as accurate as one might wish. (b) While the pH dependence of $k_{23}$ follows the ionization of a single ionizing group of the enzyme of $pK$ (app) ~ 6.6 up to pH 5.7, the values obtained for $k_{23}$ above pH 5.7 do not provide such conclusive evidence for the origin of this pH dependence. The data in Table IV show that values for $k_{23}$ at pH 6.0 are the same as, if not lower than, the $k_{23}$ values at pH 5.7. (c) The proflavin-displacement method clearly establishes the presence of a steady state intermediate (such as $EP_3$ in Equation 11) in the chymotrypsin-catalyzed hydrolysis of a specific substrate ester, but the presence of additional intermediates not shown in Equation 11 is not excluded. No evidence is obtained about the step in which $P_1$ (ethanol when ATE is the substrate) is liberated. (d) The assumption that proflavin is a competitive inhibitor at all pH values at which the reaction steps were investigated and that proflavin does not perturb the system in some as yet unknown way is implicit in the evaluation of the data; but recent experiments of Bernhard et al. (23) suggested to these authors the possibility of ternary complexes consisting of enzyme, dye, and cinnamoyl imidazole.

The present studies of individual steps in the chymotrypsin-catalyzed hydrolysis of specific substrates do, however, contribute some definite information. Direct evidence is obtained that in the catalytic hydrolysis of specific substrate amides, $K_m$ (app) is a direct measure of an enzyme-substrate dissociation constant, $K_s'$. These results, obtained from spectrophotometric measurements by the proflavin-displacement method, are in agreement with experiments reported in an accompanying paper (18), in which perturbation of the enzyme chromophore at 290 nm is used to measure a dissociation constant for the complex between the specific amide substrate APA and chymotrypsin. The spectrophotometric measurements at 290 nm (18) show that the intermediate responsible for this perturbation of the ultraviolet absorption spectrum of chymotrypsin is formed in less than 3 msec; and the present stopped flow experiments show that the intermediate observed in the proflavin experiments with specific amide substrates is formed in less than 5 mesc. Since the rate-limiting step in the chymotrypsin-catalyzed hydrolysis of these two amide substrates has a rate constant less than 0.01 set?/sec, it follows (18) that in the catalytic hydrolysis of the specific amide substrates APA and ATA, the rate-limiting step follows the formation of the ES complex and not the formation of a subsequent intermediate such as is observed in the chymotrypsin-catalyzed hydrolysis of ATE.
In the case of the specific substrate ester ATE, direct evidence is obtained in the studies presented here that the rate-limiting step does not follow the formation of the first ES complex. In these experiments, the proflavin technique permitted direct observation (Fig. 3) of the build-up of an intermediate such as $EP_2$ in Equation 11 to a steady state level, and the subsequent decay of this intermediate as the substrate is exhausted. Furthermore, analysis of this pre-steady state portion of the reaction according to Equation 20 allowed determination of $K'_s$ and $k_{z3}$. As can be seen in Table IV, the values determined for $K'_s$ are considerably larger than the values of the steady state kinetic parameter $K_m$ (app), where such values are available for comparison, and they are of the same order as the $K'_s$ values for the corresponding specific substrate amide (Table III). The values of $k_{z3}$ are generally 50 to 40 times larger than the corresponding $k_{cat}$ values, indicating that, in terms of Equation 11, $l_{coat}$ is effectively measuring $k_{cat}$. The use of Equations 22 and 23 permits calculation of values for $K$, $(app)$ from the values of $k_{z3}$ determined in these experiments and the $k_{cat}$ values reported for steady state experiments (37). These calculated values, presented in the last column of Table IV, can be seen to agree with the kinetically determined values of $K_m$ (app). The experiments presented constitute, therefore, direct evidence for a three-step mechanism, and the first direct determination of $K'_s$ and $k_{z3}$ in chymotrypsin-catalyzed hydrolysis of specific substrate esters. We have previously published the first direct determination of $K'_s$ values in the chymotrypsin-catalyzed hydrolysis of a specific substrate amide (18, 26).

REFERENCES

Investigations of the Chymotrypsin-catalyzed Hydrolysis of Specific Substrates: III. DETERMINATION OF INDIVIDUAL RATE CONSTANTS AND ENZYME-SUBSTRATE BINDING CONSTANTS FOR SPECIFIC AMIDE AND ESTER SUBSTRATES
Karl G. Brandt, Albert Himoe and George P. Hess


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