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*

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SUMMARY

Enzyme-substrate binding constants, \( K'_{es} \), for chymotrypsin and specific amide (N-acetyl-L-tryptophanamide and N-acetyl-L-phenylalaninamide) and ester (N-acetyl-L-tryptophan ethyl ester) substrates have been measured by a proflavin-displacement method. Also, the rate constant for the formation of one intermediate in the chymotrypsin-catalyzed hydrolysis of the ester has been determined at selected pH values. These constants have not previously been determined.

The proflavin-displacement method used to measure the enzyme complex concentration has previously been found to yield results in agreement with those obtained by measuring the spectral changes of the enzyme at 290 nm. These spectral changes at 290 nm, observed in all chymotrypsin-catalyzed reactions studied, have been shown to arise during the reversible formation of an enzyme-substrate complex which precedes the bond-breaking step.

New information important to an understanding of chymotrypsin-catalyzed hydrolysis in general has been obtained from this work. It is shown directly, for the first time, that in the chymotrypsin-catalyzed hydrolysis of at least two specific amide substrates, the rate limiting bond-breaking step follows the reversible formation of an enzyme-substrate complex with a dissociation constant \( K'_{es} \), and that \( K'_{es} \) and the steady state kinetic parameter \( K_{m} \) (app) are equivalent.

The pH-dependent rate constant (\( k_{23} \)) measured in the chymotrypsin-catalyzed hydrolysis of the ester we have studied is shown to pertain to a second intermediate detected in this reaction, an intermediate not seen in the chymotrypsin-catalyzed hydrolysis of the specific substrate amides.

The \( K'_{es} \) values at pH 8.0 for the complexes of \( \alpha \)-chymotrypsin with N-acetyl-L-tryptophanamide and N-acetyl-L-phenylalaninamide were found to be 4.7 mM and 29 mM, respectively, in excellent agreement with the steady state kinetic parameter \( K_{m} \) (app). In the case of the \( \alpha \)-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester, data on the pH dependence of \( k_{23} \) 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 \( k_{23} \) 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 \( K_{m} \) (app) has been reported as ~0.08 mM. Values of \( K_{s} \) and \( k_{23} \), determined in these experiments, together with previously determined values of the steady state kinetic parameter, \( k_{cat} \), allowed a calculation of \( K_{m} \) (app). At pH 5.0, this calculated value is ~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 (2–10), 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 nm (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, 16, 17). Further information was obtained from kinetic investigations (8, 9) of the physical changes of the enzyme which accompany its stoichiometric reaction with disopropyl 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 static equilibrium investigations of the spectral changes of the enzyme near 290 mµ, 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, $K'_s$, 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 absorbance of these residues in the 290 mµ region. We have therefore used another approach to the determination of $K'_s$ 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-diaminoacridine) by the enzyme.

The competitive inhibition of chymotrypsin by proflavin, first reported by Wallace, Kurtz, and Niemann (19), has been used by Weiner and Koehlund (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 tryptophan and chymotrypsin (24, 25), and for the detection of transient compounds in tryptophan- and chymotrypsin-catalyzed reactions (23, 25). The magnitude of the difference spectrum, exhibiting maxima at 465 mµ, which appears when proflavin binds to chymotrypsin, is proportional to the amount of enzyme-proflavin complex (22, 23). Introduction into the system of a substrate which competes with proflavin for the enzyme decreases the amount of enzyme-proflavin complex. Also, the substrate itself forms a weak complex with proflavin which absorbs at 465 mµ. 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 absorption coefficients of the various proflavin complexes. Since the constants pertaining to the enzyme- and substrate-proflavin complex can be determined independently, the chymotrypsin-substrate dissociation constant, $K'_s$, 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. δ-Chymotrypsin was prepared just before each use by activating chymotrypsinogen with trypsin under conditions known to yield essentially the δ form of the enzyme (27). Enzyme concentrations were determined spectrophotometrically at 280 mµ with use of a molar extinction coefficient of 50,000 m−1 cm−1 (28). 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 $[\alpha]_D^{20} +19.1 \pm 1.2$ (c. 1, in methanol); N-acetyl-L-phenylalaninamide, Lots K-4782 and K-43631, chromatographically pure, with melting point of 184–185° and $[\alpha]_D^{20} +31.1$ (c. 1.5, in methanol); and N-acetyl-D-phenylalaninamide, Lot K 3150, were obtained from Cyelo 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 mµ of 37,900 m−1 cm−2, were obtained 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 mµ region at room temperature, 24° ± 0.3.** Since these proflavin-containing solutions showed maxima at 465 mµ (22, 23), and since the absorption maximum for proflavin alone is at 444 mµ, the difference in optical density readings between 465 mµ and 440 mµ, designated $\Delta \varepsilon_{465-440}$, 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
Profavlin Concentration (pLM) vs. Optical Density at 444 nm

**Fig. 1.** Profavlin concentrations against optical density at 444 nm. 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.

The reactants and buffer. Enzyme stock solution concentrations were 5 mg per ml for the enzyme-substrate-profavlin measurements and 10 mg per ml for the enzyme-profavlin measurements. These enzyme stock solutions were prepared in 0.01 M acetate buffer (pH 5.0) for the experiments at pH levels 8.0 to 4.0, and in 0.3 M KCl adjusted to pH 2.5 with concentrated HCl for the experiments at pH 2.5; they were stored at 0°C. Stock solutions of substrate were 10 mM in concentration for ATA, and 20 to 40 nM for APA. Solutions of profavlin sulfate, 400 μM, were prepared fresh daily in boiled water which had been cooled under nitrogen and were protected from the light. The experimental solutions were prepared by mixing the appropriate amounts (see Tables I, II, and III) of reactant solutions with buffer solutions of composition such that each final solution in the pH 8.0 to 5.7 region was 0.1 M in potassium phosphate, each solution in the pH 5.4 to 4.0 region was 0.1 M in sodium acetate, and all solutions had sufficient KCl to give an ionic strength of 0.39. The correct pH level was obtained by prior adjustment of the buffer solution by addition of concentrated KOH or HCl. Since acridine dyes are known to associate at rather low concentrations (30), and significant deviations from Beer's law were observed with solutions of concentration greater than 0.05 μM (see Fig. 1), all experiments were carried out with final concentrations of profavlin no greater than 70 μM.

For the enzyme-profavlin systems, spectrophotometric traces were obtained for sample solutions containing enzyme and profavlin 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 profavlin 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 profavlin (see Table I for solution compositions). Typical results are shown in Fig. 2.

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

For the enzyme-substrate-profavlin systems, positive difference spectra were obtained by making measurements with solutions containing enzyme and profavlin in the sample beam and solutions containing substrate in addition to the same concentrations of enzyme and profavlin 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

**Fig. 2.** Tracing of Cary model 14 spectrophotometer recordings of difference spectra between α-chymotrypsin plus profavlin against profavlin. 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. Profavlin 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 9% for 40 \( \mu \text{M} \alpha\)-chymotrypsin and 1.0 mM ATA at pH 8.0, and 2% for 41 \( \mu \text{M} \alpha\)-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 Ester and Investigations of Pre-steady State for 41 PM \( \alpha\)-chymotrypsin and 2.0 mM APA at pH 8.0.**

For 40 \( \mu \text{M} \alpha\)-chymotrypsin and 1.0 mM ATA at pH 8.0, the 4 min, neglecting proflavin inhibition, are calculated to be 9% for 40 \( \mu \text{M} \alpha\)-chymotrypsin and 1.0 mM ATA at pH 8.0, and 2% in these experiments, the percentages of substrate hydrolyzed in the presence of proflavin reduces the amount of substrate hydrolysed by about one-third.

**Determination of Enzyme-Substrate Dissociation Constants for Specific Substrate Ester and Investigations of Pre-steady State for 41 PM \( \alpha\)-chymotrypsin and 2.0 mM APA at pH 8.0.**

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-substrate complex was then followed in the stopped flow apparatus at 465 nm.

Values of \( k_{obs} \) were determined at the various substrate concentrations as discussed under “Results” (see Equation 19). Values of \( K_{EF} \), 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 \( k_{obs} \) obtained as \( k_{n31} \) in steady state kinetic experiments, the constants \( k_{n31} \) and \( K' \) 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”). The time-dependent change in concentration of the enzyme-substrate complex was then followed in the stopped flow apparatus at 465 nm.

**RESULTS**

**Determination of Dissociation Constants of Chymotrypsin-specific Substrate Amide Complexes**

The calculation of \( K' \) for a specific substrate amide depends on the following conditions and assumptions. (a) \( S_0 >> E_0, F_0 \), so that \( S \approx S_0 \). 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 (90-24), 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 the 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 9% for 40 \( \mu \text{M} \alpha\)-chymotrypsin and 1.0 mM ATA at pH 8.0, and 2% for 41 \( \mu \text{M} \alpha\)-chymotrypsin and 2.0 mM APA at pH 8.0. The presence of proflavin reduces the amount of substrate hydrolysed by about one-third.


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**RESULTS**

**Determination of Dissociation Constants of Chymotrypsin-specific Substrate Amide Complexes**

The calculation of \( K' \) for a specific substrate amide depends on the following conditions and assumptions. (a) \( S_0 >> E_0, F_0 \), so that \( S \approx S_0 \). 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 (90-24), 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

\[
E_0 = E + ES + EF_S
\]

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

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

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

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

Therefore, determination of \( EF_S \) at various initial substrate concentrations allows computation of \( K' \) 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

\[
\Delta A_{\text{diff}} = (EF_S) (\Delta A_{\text{diff}})_{SF} - (EF_S) (\Delta A_{\text{diff}})_{EF} - (SF) (\Delta A_{\text{diff}})_{SF}
\]

where \( \Delta A_{\text{diff}} \) is the experimental difference spectrum measurement (see “Experimental Procedure”), \( E \) 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 \( E \) and \( SF \), which can be calculated as

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

\[
SF = \frac{S_0 - (EF_S)}{F_0 + K_{EF}}
\]

**Evaluation of Equations 6 and 7 requires a 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 \( \Delta A_{\text{diff}} \) 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, \( K_{EF} \) represents either \( K_{EF} \) or \( K_{SF} \), and the subscript zero indicates initial analytical concentration.

\[
K_{AP} = \frac{(nA_0)(C_0)}{C}
\]

where \( n \) represents the number of binding sites per molecule of \( A \). When \( A_0 \gg C \)
\[
\frac{1}{\Delta_0} = \frac{n}{K_{AP}} \left( \frac{F_0}{C} - 1 \right) = \frac{\Delta \epsilon_f}{K_{AP}} \left( \frac{n}{\Delta A^{0.144 \text{ cm}^{-1}}} \right) - \frac{1}{K_{AP}}
\]

and when \( F_0 > C \)

\[
\frac{1}{F_0} = \frac{n\Delta_0}{C} \left( \frac{C}{\Delta A^{0.144 \text{ cm}^{-1}}} \right) - \frac{1}{K_{AP}}
\]

From Equations 9 and 10 it can be seen that a plot of \( A^{-1} \) or \( F_0 \) against \( \Delta A^{0.144 \text{ cm}^{-1}} \) in the manner of Benesi and Hildebrand (35) should give a straight line from which \( K_{AP} \) (which is either \( K_{AP} \) or \( K_{SP} \)) and \( \Delta \epsilon_f \) can be determined.

In the present work, the calculations of \( E_F, S_F, E_F\), \( K_{AP} \), and \( K_{SP} \) were carried out by computer, with use of \( K_{AP} \) values determined graphically. A program using Equations 4, 5, 6, 7, and 9 was written, and values of \( K_{SP} \), each substrate concentration were obtained. In the calculations of \( K_{SP} \), a value of 2.6 \( \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \) was used for \( \Delta \epsilon_f \); this value was determined from static measurements of \( a \)-chymotrypsin-proflavin complexes 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 \( K_{SP} \) 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 \( a \)-chymotrypsin and ribuluvin also (36). The data in Table I also show that the binding of proflavin by \( a \)-chymotrypsin decreases at low pH.

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

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

\[\begin{array}{|c|c|}
\hline
\text{Material} & K_{SP} \\
\hline
\text{ATA} & 4.9 \pm 0.2 \\
\text{APA} & 2.7 \pm 0.1 \\
\text{D-APA} & 2.3 \pm 0.4 \\
\text{L-APA} & 4.4 \pm 0.4 \\
\text{D-APA} & 4.3 \pm 0.4 \\
\text{L-APA} & 3.3 \pm 0.2 \\
\text{ATA at pH 2.5} & 3.9 \pm 0.2 \\
\text{ATA at pH 4.6} & 7.3 \pm 0.5 \\
\text{ATA at pH 5.7} & 7.9 \pm 0.4 \\
\end{array}\]

\[\begin{array}{|c|c|}
\hline
\text{System} & K_{SP} \\
\hline
\text{ATA (2.5 to 14.0 \times 10^{-4} \text{ M})} & 3.2 \\
\text{ATA (2.0 to 10.5 \times 10^{-4} \text{ M})} & 4.5 \\
\text{ATA (1.0 to 7.1 \times 10^{-4} \text{ M})} & 6.7 \\
\text{ATA (5.0 to 10^{-4} \text{ M})} & 3.6 \\
\text{ATA (4.0 \times 10^{-4} \text{ M})} & 6.0 \\
\text{ATA (4.0 \times 10^{-4} \text{ M})} & 8.3 \\
\end{array}\]
TABLE III
Dissociation constants, $K'_s$, for chymotrypsin-specific amide
substrate complexes

Results obtained by the proflavin-displacement method under
steady state conditions are compared to $K_m$ (app) values deter-
mined previously. Temperature was 24°C. The concentration of
proflavin was $4.0 \times 10^{-4} \text{M}$ throughout the proflavin dis-
placement experiments.

<table>
<thead>
<tr>
<th>System</th>
<th>No. of measurements</th>
<th>$K'_s$ ($\times 10^9$ M$^{-1}$)</th>
<th>$K_m$ (app)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Chymotrypsin (4.0 × 10$^{-4}$ m)-ATA (1.5 to 10.5 × 10$^{-4}$ m), pH 8.0</td>
<td>12</td>
<td>4.7 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>α-Chymotrypsin-ATA, pH 7.9</td>
<td></td>
<td>4.2 ± 0.4 (33)</td>
<td></td>
</tr>
<tr>
<td>α-Chymotrypsin (4.0 × 10$^{-4}$ m)-ATA (1.0 to 10.5 × 10$^{-4}$ m), pH 5.7</td>
<td>19</td>
<td>6.3 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>α-Chymotrypsin-ATA, pH 5.7</td>
<td></td>
<td>3.3 ± 0.3 (39)</td>
<td></td>
</tr>
<tr>
<td>α-Chymotrypsin-ATA, pH 6.0</td>
<td></td>
<td>5.4 ± 0.9 (33)</td>
<td></td>
</tr>
<tr>
<td>α-Chymotrypsin (3.9 to 11.3 × 10$^{-4}$ m)-ATA (1.0 to 7.1 × 10$^{-4}$ m), pH 2.5</td>
<td>18</td>
<td>14.4 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>β-Chymotrypsin (2.0 × 10$^{-5}$ and 4.0 × 10$^{-4}$ m)-ATA (1.0 to 10.5 × 10$^{-4}$ m), pH 8.0</td>
<td>18</td>
<td>3.9 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>β-Chymotrypsin-ATA, pH 8.0</td>
<td></td>
<td>1.8 ± 0.1 (33)</td>
<td></td>
</tr>
<tr>
<td>α-Chymotrypsin (4.1 × 10$^{-4}$ m)-APA (2.0 to 21.2 × 10$^{-4}$ m), pH 8.0</td>
<td>18</td>
<td>28 ± 7</td>
<td></td>
</tr>
<tr>
<td>α-Chymotrypsin (4.0 × 10$^{-4}$ m and 4.2 × 10$^{-4}$ m)-APA (2.6 to 10.5 × 10$^{-4}$ m), pH 8.0</td>
<td>13</td>
<td>6.6 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>α-Chymotrypsin-n-APA, pH 7.9</td>
<td></td>
<td>12 ± 3&lt;sup&gt;b&lt;/sup&gt; (38)</td>
<td></td>
</tr>
<tr>
<td>α-Chymotrypsin-n-APA, pH 8.0</td>
<td></td>
<td>9.0 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> A concentration of $2.0 \times 10^{-4}$ M for proflavin was also used.
<sup>b</sup> $K'_s$ measured by inhibition studies of the α-chymotrypsin-
catalyzed hydrolysis of chloroacetyl-7-tyrosinamide by n-APA.
<sup>c</sup> $K'_s$ measured from spectral changes of the enzyme near 290

amide substrates are in agreement with previous steady state
kinetic measurements of $K_m$ (app).

Stopped Flow Experiments with ATE to Determine $K'_s$ and $k_{2a}$—
As reported previously (26), the results of stopped flow exper-
iments 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-
scope traces such as the one shown in Fig. 3. The differences
between such traces obtained with specific substrate esters and
the traces obtained with amides may be considered in terms of
an equation which was originally shown (40, 41) to apply to the
chymotrypsin-catalyzed hydrolysis of p-nitrophenyl acetate:

$$R + S \xrightleftharpoons k_{\text{m}} P_1 \xrightarrow{r} \text{EP}_1 \xrightleftharpoons k_{\text{m}} R + P_1$$

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 observ-
able 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 min 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 $K'_s$ diffi-
cult. 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 $EF$ concentra-
tion (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, $S_0$ is seen as a decrease in $EF$.
For the particular experiment shown in Fig. 3, this step has a
time-half 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 $EF$ concentration
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 con-
sidered to be due to the decompositions 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 $k_{2a}$ 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 sub-
sequent progress of the reaction can be measured by observing the
time-dependent change in $EF$ concentration. (b) $S_0 < S_0 < S_0$. 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 experi-
mental conditions used, the concentration of $F$ changes by not
more than 8% during the development of the steady state.
(c) $k_{2a} < k_{2a}$. This assumption is warranted by the fact that
the presence of a steady state intermediate can be detected in
traces such as Fig. 3 and that the steady state kinetic parameter,
TABLE IV

<table>
<thead>
<tr>
<th>pH</th>
<th>ATE concentration</th>
<th>k \text{a}</th>
<th>k'_{\text{a}}</th>
<th>k_{\text{cat}}</th>
<th>K_{\text{m(app)}}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MM</td>
<td>sec⁻¹</td>
<td>m \times 10⁶</td>
<td>sec⁻¹</td>
<td>m \times 10⁶</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>8.3 (37)</td>
<td>8.3 (37)</td>
</tr>
<tr>
<td>5.4</td>
<td>0.5-2.7</td>
<td>103 ± 13</td>
<td>2.0 ± 0.3</td>
<td>6.6 (37)</td>
<td>6.6 (37)</td>
</tr>
<tr>
<td>5.0</td>
<td>0.5-2.25</td>
<td>35 ± 9</td>
<td>2.3 ± 0.6</td>
<td>5.6</td>
<td>5.6</td>
</tr>
<tr>
<td>4.6</td>
<td>0.6-2.75</td>
<td>17.5 ± 1.3</td>
<td>2.9 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.3</td>
<td>0.6-2.75</td>
<td>8.2 ± 1.2</td>
<td>3.0 ± 0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The experimentally observed values $K'_{\text{a}}(1 + (P_0/K_{\text{B}}F))$ (see Equation 20) were converted to $K'_{\text{a}}$ by assuming an average value for $K_{\text{B}}F$ of $4.4 \times 10^{-3}$ m at all pH values.

$k_{\text{cat}}$ is small compared to $k_{\text{obs}}$ in Equation 16a. The steady state kinetic constant $k_{\text{cat}}$ can therefore be considered a measure of $k_{\text{a4}}$.

(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).

$$K_{\text{EF}} = \frac{[E][F]}{EF}$$

or

$$E = K_{\text{EF}} \frac{F}{E}$$

$$ES = \frac{[E][S]}{K'_{\text{a}}} = EF \frac{K_{\text{EF}}S_0}{F_0K'_{\text{a}}} = EF_{\text{a}}$$

Since $E_0 = E + ES + EP_2 + EF$

$$EP_2 = E_0 - EF \left( \frac{K_{\text{EF}}}{F_0} + \frac{K_{\text{EF}}S_0}{F_0K'_{\text{a}}} + 1 \right) = E_0 - EP_{\text{a}}$$

$$\frac{d(EP_2)}{dt} = k_{31}S + k_{32}EP_2$$

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

$$\left( \frac{EF}{t} \right)_t = \frac{E_0}{k_{31}F_0 + k_{32}A} \left[ k_{31} + \frac{k_{32}}{F_0} \frac{S_0^{k_{32}}}{e^{k_{32}}} \right]$$

FIG. 4. Stopped flow data at 465 nm for ATE added to a mixture of α-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 $k_{\text{obs}}$. Final solution concentrations are 10 μM α-chymotrypsin and 50 μM proflavin; ionic strength = 0.39; temperature = 28°C. ○, pH 5.4; initial concentration of ATE solution ($S_0$) = 2.70 mM; $k_{\text{obs}} = 38.7$ sec⁻¹. ●, pH 5.4, $S_0 = 0.90$ mM; $k_{\text{obs}} = 19.9$ sec⁻¹. △, pH 4.6, $S_0 = 2.75$ mM; $k_{\text{obs}} = 5.70$ sec⁻¹.
is the molar absorption coefficient of the enzyme-proflavin complex at 465 mμ. Therefore, a plot according to

\[
\ln [(A_{465})_t - (A_{465})_{ss}] = C - k_{obs} t
\]  

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 \( k_{obs} \), the slope, 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

\[
k_{obs} - k_{34} = k_{33} - \left[ \frac{k_{obs} - k_{33}}{K'_{S}} \right] 
\]

Since \( F_0 \), \( K_{EF} \), and \( k_{34} \) are known, \( k_{33} \) 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).

The steady state concentration of EF is obtained by use of Equations 12 and 13 and the relation \((EF)_{ss} = \frac{k_{33} ES}{k_{34} S_0}\). Substitution into Equation 14 and rearrangement gives

\[
E_0 - (EF)_{ss} = \frac{k_{33} S_0}{k_{34} S_0 + 1} 
\]

Therefore

\[
(EF)_{ss} = \frac{k_{33} E_0}{k_{34} S_0 + 1} 
\]

Then

\[
(EF)_{ss} - (EF)_{ss} = \frac{1}{k_{33}} [(A_{465})_t - (A_{465})_{ss}] 
\]

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

Fig. 6. Observed values of \( k_{33} \) 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

\[
\frac{1}{k_{33}} = \left( \frac{1}{k_{33} \text{ (lim)}} \right) (H^+) + \frac{1}{k_{33} \text{ (lim)}} 
\]

which is a rearrangement of Equation 21 in the text. These calculations for the line were made with use of a \( k_{33} \text{ (lim)} \) value of 1800 sec⁻¹ and a \( K'_{S} \) value corresponding to a controlling group of \( \text{pK (app)} = 6.6 \).
In Table IV are tabulated the values and standard deviations obtained for $K'_s$ and $k_{sa}$ 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) = $K'_s k_{34} / (k_{23} + k_{34})$ (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

$$k_{23} - k_{34} \; (\text{lim}) \left( \frac{K'_s}{K'_s + [H^+]} \right)$$

(21)

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 $k_{34}$ (lim) is the limiting, pH-independent value of $k_{34}$ reached at the pH at which the ionizing group is completely dissociated. Fig. 6, in which $k_{23}$ is plotted against $[H^+]$, 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 sec$^{-1}$ and a controlling pK (app) of 6.6; the line in Fig. 6 was calculated for these values. An ionizing group with about the same pK (app) value has been implicated in the pH dependence of all chymotrypsin-catalyzed reactions studied (37).

**DISCUSSION**

The relationships between the steady state parameters $k_{cat}$ and $K_m$ (app) and the constants $K'_s$, $k_{sa}$, and $k_{sa}$ of Equation 11 have been set forth (41). These relationships can be expressed as

$$k_{cat} = k_{sa} k_{34} / (k_{23} + k_{34})$$

(22)

$$K_m \; (\text{app}) = K'_s k_{34} / (k_{23} + k_{34})$$

(23)

It was first suggested by Gutfreund and Sturtevant (43) that $k_{23}$ is rate-limiting for specific amide substrates; this requires that $K'_s$ and $K_m$ (app) be equivalent ($k_{23} \gg k_{34}$ in Equation 23). In the case of specific ester substrates, it has been proposed (37, 44) that there is a rapid build-up of an intermediate, such as $EP_2$ in Equation 11, to a steady state level, followed by the rate-limiting decomposition of this intermediate; this requires that $K'_s$ be different from $K_m$ (app), and specifically that $K'_s$ be larger ($k_{23} \gg k_{14}$ in Equation 23).

These suggestions about the chymotrypsin-catalyzed hydrolysis of specific substrates were based entirely on steady state kinetic studies which do not permit identification of the steady state kinetic parameters, $k_{cat}$ and $K_m$ (app), with $K'_s$, $k_{sa}$, or $k_{sa}$ in Equation 11. In addition, even the validity of Equation 11, which is based on investigations of the chymotrypsin-catalyzed hydrolysis of p-nitrophenyl acetate, has recently been questioned. Fuller and Sturtevant (45) showed that in the chymotrypsin-catalyzed hydrolysis of p-nitrophenyl acetate the formation of $EP_2$ (Equation 11) gives bimolecular kinetics, and their re-evaluation of previously published data (41) on this 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 nonlinear 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 $k_{sa}$ and $K'_s$ as accurate as one might wish. (b) While the pH dependence of $k_{sa}$ follows the ionization of a single ionizing group of the enzyme of pH pK (app) ~6.6 up to pH 5.7, the values obtained for $k_{sa}$ 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 $k_{sa}$ at pH 6.0 are the same as, if not lower than, the $k_{sa}$ values at pH 5.7. (c) The.proflavin-displacement method clearly establishes the presence of a steady state intermediate (such as $EP_2$ 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 possible existence 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 msec. Since the rate-limiting step in the chymotrypsin-catalyzed hydrolysis of these two amide substrates has a rate constant less than 0.07 sec$^{-1}$, 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 profilein 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 $k_{24}$. 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 $k_{24}$ are generally 50 to 40 times larger than the corresponding $k_{cat}$ values, indicating that, in terms of Equation 11, $k_{cat}$ is effectively measuring $k_{24}$. The use of Equations 22 and 23 permits calculation of values for $K_m$ (app) from the values of $k_{24}$ and $K'_s$ 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 $k_{24}$ 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).

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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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