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Investigations of the Chymotrypsin-catalyzed Hydrolysis of Specific Substrates

IV. PRE-STEADY STATE KINETIC APPROACHES TO THE INVESTIGATION OF THE CATALYTIC HYDROLYSIS OF ESTERS*

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SUMMARY

Pre-steady state kinetic parameters pertaining to chymotrypsin-catalyzed hydrolysis of specific substrate esters can be obtained from measurements of the displacement of the competitive inhibitor proflavin from enzyme by substrate. This method, which was previously used in experiments with specific substrate amides, has now been applied to the investigation of *N*-acetyl-L-phenylalanine methyl ester, *N*-acetyl-L-tryptophan ethyl ester, and *N*-furylacryloyl-L-tyrosine ethyl ester. Results were found to be in agreement with data obtained by established procedures including measurement of spectral changes of the enzyme upon formation of complexes with substrate and direct measurement of product formation. All available data on chymotrypsin-catalyzed hydrolysis of specific substrate esters are consistent with the previously proposed mechanism

$$E + S \underset{}{\overset{K'_S}{\rightleftharpoons}} ES \xrightarrow[k_{23}]{\nearrow P_1} EP_2 \xrightarrow[k_{34}]{} E + P_2$$

in which P_1 represents an alcohol and P_2 an acid.

Chymotrypsin-catalyzed reactions have been considered in terms of the equation:

$$E + S \underset{}{\overset{K'_S}{\rightleftharpoons}} ES \xrightarrow[k_{23}]{\nearrow P_1} EP_2 \xrightarrow[k_{34}]{} E + P_2 \quad (1)$$

where S represents substrate ester or amide, ES an enzyme-substrate complex, EP_2 an enzyme-substrate compound, P_1 an alcohol or amine, and P_2 a free acid. This equation is based on kinetic investigations of the chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate (1, 2), although in this particular reaction there is no evidence for an enzyme-substrate complex (3). In the *p*-nitrophenyl acetate reaction, EP_2 in Equation 1 has been identified as a chymotrypsin molecule in which a single serine hydroxyl group has been acetylated (4).

Kinetic investigations of the chymotrypsin-catalyzed hydrolysis of specific substrates—ester and amide derivatives of aromatic amino acids—have mainly consisted of steady state measurements (5), which yield, for the simple mechanism shown in Equation 1, only combinations of rate and equilibrium constants (2):

$$\frac{V_{\max}}{E_0} = k_{\text{cat}} = \frac{k_{23}k_{34}}{k_{23} + k_{34}} \quad (2)$$

where the subscript zero denotes initial concentration;

$$K_m \text{ (app)} = K'_S \frac{k_{34}}{k_{23} + k_{34}} \quad (3)$$

and thus:

$$\frac{k_{\text{cat}}}{K_m \text{ (app)}} = \frac{k_{23}}{K'_S} \quad (4)$$

Recently we have started to investigate (6–12) the pre-steady state kinetics of the chymotrypsin-catalyzed hydrolyses of both amides and esters of aromatic amino acids, the specific substrates of the enzyme. A variety of methods has been used. These include measurement of spectral changes of the enzyme upon formation of complexes with substrates, direct measurement of product formation, and measurement of the displacement of the competitive inhibitor proflavin from enzyme by substrate. Spectrophotometric and stopped flow techniques permit the evaluation of pre-steady state rate and equilibrium constants in addition to the over-all constants obtainable by conventional steady state methods. Our results, although showing differences between amide and ester substrates in the relative rates of different reaction steps, have so far proved to be consistent with the mechanism of Equation 1.

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Recently, however, the possibility that there are additional intermediates (between ES and EP_2 in Equation 1) was suggested by the results of experiments of Barman and Gutfreund (13) on the chymotrypsin-catalyzed hydrolysis of Fa-Tyr-OEt.[1] These authors inferred that liberation of ethanol (P_1 in Equation 1) is slower than the formation of the enzyme-substrate compound (EP_2). The possibility that proflavin may not be a competitive inhibitor has also been raised (14), bringing into question the usefulness of the proflavin displacement method for determination of pre-steady state rate and equilibrium constants. It is important to know about the applicability of the proflavin displacement method, since it is the only method so far found that can be used with all chymotrypsin substrates.

The experiments reported here were designed to evaluate further the usefulness of the proflavin displacement method in investigations of chymotrypsin-catalyzed hydrolyses. Comparisons were made of results obtained by this and other methods. We investigated the pre-steady state kinetics of the chymotrypsin-catalyzed hydrolysis of Ac-Phe-OMe, following the reaction not only by the proflavin displacement technique (6, 9, 10, 14–17) but also by an independent method based on spectral changes of the enzyme at 290 mμ (18–21). We also made comparative measurements of Ac-Trp-ONp at three pH levels, measuring both *p*-nitrophenol release and proflavin displacement. Finally, we investigated the pre-steady state kinetics of the α-chymotrypsin-catalyzed hydrolysis of Fa-Tyr-OEt, in order to assess the suggestion of Barman and Gutfreund that in this reaction alcohol (P_1 in Equation 1) is not released concomitantly with the formation of EP_2.

EXPERIMENTAL PROCEDURE

Materials and Apparatus

Three times crystallized, salt-free α-chymotrypsin (Lots CDI-6164, CDI-6168–9, and CDI-6KB) was obtained from Worthington. Enzyme concentration was determined spectrophotometrically at 280 mμ with the use of a molar extinction coefficient of 50,000 M^{-1} cm^{-1} (22). A molecular weight of 25,000 (23) was assumed.

Fa-Tyr-OEt, Lot K-5621, with melting point of 134–137° and a molar extinction coefficient at 304 mμ of 26,500 M^{-1} cm^{-1}, was obtained from Cyclo. Ac-Phe-OEt, Lot G 2441, with melting point of 89–90°, Ac-Phe-OMe, Lot K-4778, and proflavin sulfate, Lot M 2200, with a molar extinction coefficient at 444 mμ of 37,900 M^{-1} cm^{-1}, were obtained from Mann. Ac-Trp-ONp was prepared as described below. All other reagents were reagent grade, obtained from Mallinckrodt.

A Cary model 14 recording spectrophotometer with 1-cm silica cells and 0 to 2.0 or 0 to 0.2 slide wires was used for the spectrophotometric measurements. Determinations of pH were made on a Radiometer pH meter, type TTT1c, with reference to Beckman pH 7.0 and pH 4.0 standard buffers. For the stopped flow experiments, a Gibson-Durrum stopped flow spectrophotometer was used. This instrument has cells with 2-cm light path or 5-mm light path and a tungsten-iodide light source with grating monochromator. Time-dependent change in light transmission of the experimental solutions was recorded on a Tektronix 564 storage oscilloscope or on a Tektronix 545B oscilloscope. A Radiometer pH-stat was used for some steady state kinetic measurements of ester hydrolysis. An analogue computer, model EA1-TR-48, of Electronics Associates, was used in processing some of the data.

Preparation of Ac-Trp-ONp—A 1.03 g (0.0022 mole) of benzyloxycarbonyl-L-tryptophan *p*-nitrophenyl ester, obtained from Cyclo (Lot K-5082, with melting point of 95–98°), was dissolved in 10 ml of glacial acetic acid saturated with HBr, and the mixture was allowed to stand at room temperature for 30 min. After precipitation of the product by the addition of 100 ml of anhydrous ether in small portions, the supernatant liquid was decanted and the precipitate was washed with small portions of anhydrous ether (twice) and cold water (once). The precipitate was dried under reduced pressure to yield 0.56 g (62% yield) of crystalline L-tryptophan *p*-nitrophenyl ester hydrobromide with melting point of 185–190°. This material was used without further purification in the subsequent step. In a mixture of 0.75 ml (0.007 mole) of acetic anhydride in 10 ml of ethyl acetate and 15 ml of 0.1 M phosphate buffer (pH 7.0), there was suspended 2.3 g (0.006 mole) of the L-tryptophan *p*-nitrophenyl ester hydrobromide. The reaction mixture was stirred for 30 min at room temperature, and then the organic layer was removed, dried over drierite, and filtered. Solvent was then removed under reduced pressure, and the residue was dissolved in a small amount of benzene. Addition of hexane yielded an oil which crystallized on standing to 0.6 g (29% yield) of *N*-acetyl-L-tryptophan *p*-nitrophenyl ester (Ac-Trp-ONp), with melting point of 127–132° and $[\alpha]_D^{24}$ −41° (*c*, 1, chloroform). This material was recrystallized from hexane-benzene before use in the experiments; it was at least 95% optically pure, as judged by the liberation of *p*-nitrophenol in the α-chymotrypsin-catalyzed hydrolysis. An additional recrystallization of this compound from acetone-ether yielded an analytically pure material of melting point 131–133°.

$C_{19}H_{17}N_3O_5$

Calculated: C 62.13%, H 4.66%, N 11.44%
Found[2]: C 62.04%, H 4.65%, N 11.35%

A recent report (24) gives a value of $[\alpha]_D^{20}$ −46.3° (*c*, 1, chloroform) for this compound, and a melting point of 130–131.5°.

Methods

Spectrophotometric Experiments with Ac-Phe-OMe—Measurements of proflavin displacement and of spectral changes of chymotrypsin near 290 mμ (ΔD_{290}) as a function of time were made at pH 3.1 or 3.2 and 25.0°.

For the ΔD_{290} measurements, the procedure was as follows. Two milliliters of a solution of substrate (20 mM to 3 mM in mM HCl) with ionic strength of 0.4 with KCl were pipetted into the thermostated quartz spectrophotometer cell. The reaction was initiated by addition, with a plastic plunger device, of 0.1 ml of 0.8 mM α-chymotrypsin in mM HCl. Concentrations in the final solutions were therefore 37 μM α-chymotrypsin and 19 mM to 2.9 mM Ac-Phe-OMe. As soon as the addition was completed and the sample cell cover was closed, recording of the optical density at 288.5 mμ was begun; time between addition of enzyme and the start of recording was about 5 sec. A neutral density filter was in place in the reference beam at all times, and the 0 to

[1] The abbreviations used are: Fa-Tyr-OEt, furylacryloyl-L-tyrosine ethyl ester; Ac-Phe-OMe, *N*-acetyl-L-phenylalanine methyl ester; Ac-Trp-ONp, *N*-acetyl-L-tryptophan *p*-nitrophenyl ester; Ac-Phe-OEt, *N*-acetyl-L-phenylalanine ethyl ester.

[2] Performed by Scandinavian Microanalytical Laboratory.

0.2 slide wire was used. In some of the runs, a buffer 0.1 M in glycine-HCl and 0.3 M in KCl was used to maintain the pH; in all cases, exact pH levels were established by measurement of duplicate solutions on the pH meter.

The experiments in which the displacement of proflavin was measured were carried out in exactly the same way as the ΔD_{290} experiments, except that observations were made at 465 mμ, and the reaction was initiated by the addition of 0.1 ml of a mixture containing mM proflavin and 0.2 mM α-chymotrypsin. Concentrations in the final solutions were therefore 9.5 μM α-chymotrypsin, 47.5 μM proflavin, and 19 mM to 2.9 mM Ac-Phe-OMe. In the calculations, a value of 0.1 mM was used for K_{EF}, the dissociation constant of the enzyme-proflavin complex: this value was obtained by interpolation of values measured at pH 2.5 (K_{EF} = 0.14 mM) and pH 4.0 (K_{EF} = 0.08 mM) (10).

Stopped Flow Experiment with Ac-Phe-OMe—Proflavin displacement during the α-chymotrypsin-catalyzed hydrolysis of Ac-Phe-OMe in the presence of proflavin was measured by the stopped flow technique at pH 6.0. A solution containing enzyme and proflavin was mixed in the apparatus with a solution of equal volume containing substrate and measured in the cell with a 5-mm light path. Initial concentrations in the reactant solution were 10 μM α-chymotrypsin, 2.0 mM Ac-Phe-OMe, and 50 μM proflavin. The solution contained 0.1 M phosphate buffer and had an ionic strength of 0.4. The temperature was 25.2 ± 0.5°. The time-dependent change in concentration of the enzyme-proflavin complex was followed at 465 mμ and recorded on a storage oscilloscope.

Steady State Kinetic Measurements with Ac-Phe-OEt—These measurements were performed at pH 5.0 and 25.5° in 0.4 M KCl by continuous titration of the liberated acid on a Radiometer pH-stat (25). Initial rates were determined at an enzyme concentration of about 1 μM and at several substrate concentrations in the range 0.2 to 7.0 mM. The kinetic constants k_{cat} and K_m (app) were calculated by means of a digital computer program written for the Lineweaver-Burk form of the Michaelis-Menten rate equation (26).

Stopped Flow Experiments with Ac-Trp-ONp—Measurements of proflavin displacement and of p-nitrophenol release were made at pH levels 2.5, 3.5, and 4.3 at 26.5 ± 0.5°. Solutions containing α-chymotrypsin and proflavin, or just the enzyme, were mixed according to a standard stopped flow procedure (in the 2-cm cells) with solutions of equal volume containing substrate. For the experiments at pH 2.5, the solutions were 0.05 M in KCl and the pH was adjusted with M HCl; for the experiments at pH levels 3.5 and 4.3, 0.05 M potassium acetate buffer was used in both reactant solutions. All solutions contained 1.6% acetonitrile and had an ionic strength of 0.05. Experiments were conducted under initial conditions $E_0 \gg S_0$ in the absence of proflavin, and also $S_0 \gg E_0$ in either the presence or the absence of proflavin. The initial concentrations of these components are given in Table II.

The time-dependent change in concentration of the enzyme-proflavin complex was followed at 480 mμ, and the p-nitrophenol release at 348 mμ ($\Delta\epsilon_M$ = 5450 M^{-1} cm^{-1}). Under the most unfavorable conditions, the contribution of the change in enzyme-proflavin complex to the total signal change at 348 mμ was less than 10%. The p-nitrophenol release made no detectable contribution to the signal change at 480 mμ.

Steady State Kinetic Measurements with Ac-Trp-ONp at pH 3.5—The reaction was measured by following the release of p-nitrophenol at 320 mμ ($\Delta\epsilon$ = 9750 M^{-1} cm^{-1}) in the Cary spectrophotometer (0 to 2.0 slide wire) with cells thermostatted at 25°. Distilled water was used as a reference. The reaction was started by mixing 0.05 ml of an acetonitrile solution of substrate with 3 ml of a buffered enzyme solution. Initial enzyme concentration (E_0) in the experimental solutions was 0.2 μM and initial substrate concentrations (S_0) were 1.5, 2.5, and 4.0 μM in the various experiments. All solutions were 0.1 M in potassium acetate buffer and contained 1.6% acetonitrile. Measurements at each substrate concentration were made both in the presence and in the absence of 50 μM proflavin. Values of k_{cat} and K_m (app) were calculated as discussed under "Results."

Stopped Flow Experiments with Fa-Tyr-OEt—The α-chymotrypsin-catalyzed hydrolysis of Fa-Tyr-OEt in the presence of proflavin was followed by standard stopped flow procedure at 480 mμ in a 2-cm light path cell at pH 5.0 and 24.5 ± 0.5°. Final solution concentrations were 47.5 μM α-chymotrypsin, and 305 μM to 51 μM Fa-Tyr-OEt. All solutions were 3% in acetonitrile and 0.1 M in potassium acetate to give an ionic strength of 0.1.

Steady State Kinetic Measurements with Fa-Tyr-OEt—The reaction was followed at pH 5.0 and at pH 6.6 in the Cary spectrophotometer (0 to 2.0 slide wire) at 25.0 ± 0.5° and at 325 mμ, where the difference spectrum between Fa-Tyr-OEt and the free acid is largest ($\Delta\epsilon_M$ = 1525 M^{-1} cm^{-1}). Substrate stock solution, 2 ml in volume, was pipetted into the sample cell, and the reaction was initiated by addition of 0.1 ml (for pH 5.0 reaction) or 0.01 ml (for pH 6.6 reaction) of α-chymotrypsin in mM HCl by means of a plastic plunger mixing device. The final solutions at pH 5.0 were buffered with 0.1 M sodium acetate and contained 3% acetonitrile and sufficient NaCl to bring the ionic strength to 0.1. The final solutions at pH 6.6 were buffered with 0.08 M potassium phosphate, and measurements were made both in the presence and absence of 3% acetonitrile. Initial concentrations in the final solutions were therefore: α-chymotrypsin, 0.50 μM at pH 5.0 and 0.052 μM at pH 6.6; Fa-Tyr-OEt, 31.5 μM to 118 μM at pH 5.0, 25 μM to 140 μM at pH 6.6 when 3% acetonitrile was present, and 26 μM to 104 μM at pH 6.6 in the absence of acetonitrile. It was assumed that the carboxyl group of N-furylacryloyl-L-tyrosine is completely ionized at the pH levels used. Kinetic parameters were calculated from the measured initial rates according to a digital computer program based on the Lineweaver-Burk form of the Michaelis-Menten rate equation (26).

Evaluation of k_{23}, k_{34}, and K'_S for Mechanism Shown in Equation 1—The calculation of rate constants and K'_S values for the chymotrypsin-catalyzed hydrolysis of ester substrates from stopped flow or time-dependent spectrophotometric measurements of enzyme-substrate and enzyme-substrate-proflavin systems has been discussed (6, 10). As previously shown (10), the observed rate constant for the attainment of a steady state, k_{obs}, is obtained from experiments such as shown in Fig. 1; k_{obs} can be evaluated as the slope of a line plotted according to the relation

$$\ln\,[(A)_t^\lambda - (A)_{ss}^\lambda] = C - k_{obs}t \quad (5)$$

where A is absorbance of the solution at time t and at the time when steady state, ss, is reached, and λ is the wave length at which the absorbance is measured. In the experiments reported here, λ = 288.5 mμ in the spectral change measurements, λ = 465 mμ in the proflavin displacement experiments, and λ = 320

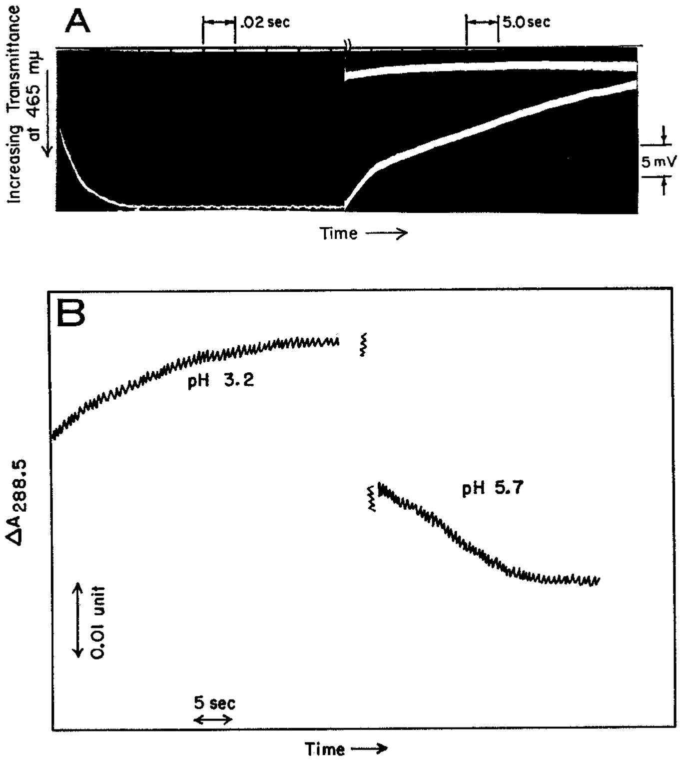

FIG. 1. *A*, photographs of oscilloscope traces in a stopped flow experiment with Ac-Phe-OMe at pH 6.0 and 25.2°. Initial solution concentrations were 2.0 mM Ac-Phe-OMe, 10 μM α-chymotrypsin, and 50 μM proflavin in 0.1 M phosphate buffer; ionic strength = 0.4 (with KCl). Measurements were made in the Gibson-Durrum stopped flow apparatus. The *upper trace* shows the second sweep of the oscilloscope. *B*, trace of the spectrophotometric measurement of the catalyzed hydrolysis of Ac-Phe-OMe as a function of time. Measurements were made at 288.5 mμ in the Cary spectrophotometer (0 to 0.2 slide wire). At a specific time during the interval in which the absorbance exhibits a constant value, the initial pH of 3.2 was changed to pH 5.7 by the addition of a small amount of base. Initial solution concentrations were 17.2 mM Ac-Phe-OMe and 37 μM α-chymotrypsin. Ionic strength was 0.4 and temperature was 25°.

mμ in the *p*-nitrophenol release measurements. A minimum of five different experiments, such as shown in Fig. 1, were run in order to establish the slope of the line.

It has been shown (10) that the parameters pertaining to Equation 1—k_{23}, k_{34}, and K'_S—can be obtained from the k_{obs} values, provided that substrate or enzyme concentrations sufficiently greater than K'_S can be obtained experimentally; otherwise, only an apparent second order rate constant, k_{23}/K'_S, can be obtained. Implicit in the evaluation of these constants are the following assumptions. (*a*) Equation 1 is the simplest equation which describes the observed progress curves of specific substrate esters. (*b*) The enzyme-substrate complex (*ES*) and, when proflavin is present, the enzyme-proflavin complex (*EF*) are formed in a rapid pre-equilibrium phase of the reaction. (*c*) Initial concentrations of substrate (*S*), enzyme (*E*), and proflavin (*F*) have the relative magnitudes of either $S_0 \gg E_0 < F_0$ or $E_0 \gg S_0$. These conditions allow the concentrations of the materials in excess to be considered constant during the period of observations and equal to their initial analytical values. Calculations indicate that, under the most unfavorable conditions used in the experiments reported here, the concentration of *F* changes by less than 8% during the development of the steady state. (*d*) $k_{34} < k_{23}$. This assumption is warranted when the presence of a steady state intermediate can be detected in traces such as those shown in Fig. 1. When the steady state kinetic parameter k_{cat} is small compared to the experimentally observed rate constant (k_{obs}) for the formation of EP_2, k_{cat} is considered a measure of k_{34}. (*e*) Proflavin is a competitive inhibitor of the enzyme and does not perturb the system in some unknown way.

The equations used for evaluating the pre-steady state kinetic parameters from k_{obs} values are given under "Appendix." Data weighting and the calculation of the standard errors of the kinetic constants were performed as discussed by Wilkinson (27).

Calculation of Progress Curves—Progress curves for intermediates and products in the α-chymotrypsin-catalyzed hydrolysis of Fa-Tyr-OEt at pH 6.6 were generated by means of an analogue computer. Conditions taken for the reaction were approximately equal initial concentrations of enzyme and substrate (E_0 = 0.27 mM and S_0 = 0.22 mM). Required values of K'_S, k_{23}, and k_{34} (taken as equivalent to k_{cat}) were obtained experimentally under the conditions $S_0 > E_0$, and are listed in Table III. The complex *ES* was considered to be formed in a rapid pre-equilibrium phase of the reaction.

RESULTS

Pre-Steady State Kinetic Experiments with α-Chymotrypsin and Ester Substrates—Comparable determinations of K'_S and k_{23} (Equation 1) were obtained from temporal measurements made either with the spectrophotometric Gibson-Durrum stopped flow apparatus or a Cary spectrophotometer, and with use of methods based on the displacement of proflavin from the enzyme by substrate, or on spectral changes of the enzyme near 290 mμ, or on release of alcohol (P_1 in Equation 1).

A typical oscilloscope trace obtained when excess ester substrate is mixed with enzyme and proflavin in the spectrophotometric stopped flow apparatus is shown in Fig. 1*A*. This trace was obtained at pH 6.0 and 465 mμ with Ac-Phe-OMe as substrate. Interpretation of a trace such as this is made on the basis of the following observations. The formation of a chymotrypsin-proflavin complex results in a perturbation, with a maximum at 465 mμ, of the proflavin absorption spectrum (14). Introduction of substrate into a system of enzyme and proflavin results in competition between proflavin and substrate for the enzyme; consequently, less proflavin is bound by the enzyme, and the absorption due to complex is decreased. Interpretation of the observable reaction steps (Fig. 1*A*) is therefore as follows. (*a*) The initial change is a rapid decrease in concentration of the enzyme-proflavin complex, *EF*. This change is usually too fast to be measured and is not shown; representation of it would have required a third time scale in Fig. 1*A*. This initial decrease is considered to reflect the formation of an enzyme-substrate complex (*ES* in Equation 1). (*b*) The second step is a second decrease in the concentration of *EF*, considered to occur as a result of the formation of another intermediate, such as EP_2 in Equation 1. This step has an observed rate which is dependent on both pH and initial substrate concentration. (*c*) Subsequent to this rapid but measurable decrease in *EF* concentration, there is a period during which essentially no change in *EF* occurs. The length of this period also depends on substrate concentration, and is considered to reflect the time during which there is maintained a steady state concentration of the intermediate such as EP_2 in Equation 1. (*d*) Finally, an increase in the concentration of EF occurs. This is considered to be due to the decomposition of EP_2.

The same reaction phases can be seen in experiments in which enzyme is mixed with excess substrate and the resulting absorption changes near 290 mμ are recorded as a function of time on the Cary spectrophotometer. Results of a typical experiment with α-chymotrypsin and Ac-Phe-OMe are represented in Fig. 1*B*. In this particular experiment, the reaction was begun at pH 3.2, and the steady state part of the reaction was shortened by the addition of a small amount of base to bring the pH to 5.7.

When experiments such as those show in Fig. 1 are performed at various initial substrate or initial enzyme concentrations, the parameters pertaining to Equation 1 (k_{23}, k_{34}, and K'_S) can be evaluated (see "Experimental Procedure").

Pre-Steady State Kinetic Investigations of α-Chymotrypsin-catalyzed Hydrolysis of Ac-Phe-OMe at pH 3.1—The constants k_{23} and K'_S for this reaction were evaluated from plots of ($k_{obs} - k_{34}$) against ($k_{obs} - k_{34})/S_0$ in accordance with Equation II-7 under "Appendix." These plots are shown in Fig. 2. The progress of the reaction was followed at 25.0° in the spectrophotometer by measuring the time dependence of the spectral changes of the enzyme near 290 mμ in solutions which did not contain proflavin (○ in Fig. 2), and also by measuring the time dependence of proflavin displacement from the enzyme (● in Fig. 2). The best value for k_{34} for both the experiments, one with proflavin and one without, was calculated by computer analysis of the data, and this k_{34} value was used in both experiments to calculate the ($k_{obs} - k_{34}$) values. The *lines* in the figure were established on the basis of a least squares computer calculation. The slopes of the lines correspond to K'_S in the ΔD_{290} experiment without proflavin and to $K'_S[1 + (F_0/K_{EF})]$ in the proflavin displacement experiment. The intercepts of the *ordinate* correspond to k_{23}. The constants evaluated from these experiments are listed in Table I.

Although the time dependence of the spectral changes of the enzyme near 290 mμ is more difficult to measure than the time dependence of the proflavin displacement, and the scatter of data obtained by the former method is therefore greater, it was possible to fit both sets of data with a single k_{34} value, and there was good agreement between the k_{23} values obtained by the two methods (see intercepts of the *ordinates* in Fig. 2, and Table I). The agreement between K'_S values is less satisfactory (see Table I). This comparison, however, is based on three independent measurements. For the spectral change experiments, the slope of a line, such as is shown in Fig. 2, is required; for the proflavin experiments, the calculation of K'_S requires not only the slope of the experimental line, but also the enzyme-proflavin dissociation constant.

Evaluation of k_{23} and k_{34} allows the calculation of a k_{cat} value (see Equation 2) and thus permits comparison of k_{cat} obtained from steady state experiments with a value calculated from the evaluated pre-steady state rate constants. We made such a comparison in the following way. We calculated a value of k_{cat} from steady state measurements of the hydrolysis of the ethyl ester of *N*-acetyl-L-phenylalanine, which exhibits the same steady state kinetic parameters as Ac-Phe-OMe. Since the k_{cat} value for esters of amino acids cannot easily be measured below pH 5.0 by the pH-stat method (25), which depends on liberation of hydrogen ions as a consequence of ester hydrolysis, we calculated k_{cat} at pH 3.1 from steady state experiments performed at pH 5.0 and 25.5°. The steady state measurements at

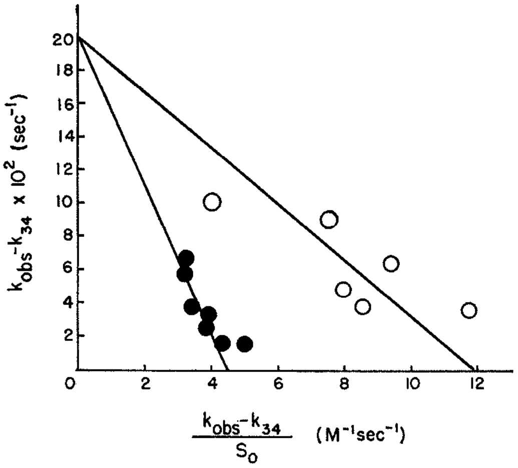

FIG. 2. The α-chymotrypsin-catalyzed hydrolysis of Ac-Phe-OMe at pH 3.1 and 25.0° under the conditions $S_0 \gg E_0$ or $S_0 \gg E_0 < F_0$ (see "Experimental Procedure"). The observed rate constant k_{obs} refers to spectrophotometric measurements of spectral changes of the enzyme at 288.5 mμ (○) and to measurements at 465 mμ of displacement of proflavin from complex with the enzyme (●). *Points* were established by a computer calculation giving the best value of k_{34} for both experiments; *lines* were drawn according to a least squares computer calculation. Concentrations in the final solutions were 37 μM α-chymotrypsin and 19 mM to 2.9 mM Ac-Phe-OMe for the spectral change measurements, and 9.5 μM α-chymotrypsin, 47.5 μM proflavin, and 19 mM to 2.9 mM Ac-Phe-OMe for the proflavin displacement experiments. Ionic strength of measured solutions was 0.4. Table I lists constants evaluated from these data.

TABLE I

Pre-steady state constants pertaining to α-chymotrypsin-catalyzed hydrolysis of Ac-Phe-OMe

Measurements of k_{23}, k_{34}, and K'_S were made at pH 3.1 and 25.0°. Ionic strength = 0.4. Designations of constants are in accordance with Equation 1.

Method of measurement	k_{23}	K'_S	k_{34}[a]	k_{cat}
	sec^{-1}	*mM*	*sec*$^{-1}$	*sec*$^{-1}$
Spectral changes of enzyme near 290 mμ[b]. . . .	0.20 ± 0.11	17 ± 8	0.03	0.03[c]
Proflavin displacement. .	0.20 ± 0.05	27 ± 6[d]	0.03	

[a] Computer value which gives the best lines (as determined by method of least squares) for data from both experiments.

[b] Actual measurements were made at 288.5 mμ.

[c] Calculated from k_{23} and k_{34} according to Equation 2. Calculated also, on the basis of control of k_{cat} by an ionizing group of the enzyme with pK(app) ~7, from a k_{cat} value of 2.5 sec^{-1} observed in the α-chymotrypsin-catalyzed hydrolysis of the ethyl ester, Ac-Phe-OEt, at pH 5.0 and 25.5° (see text).

[d] Calculated on the basis of a K_{EF} value of 0.1 mM (10).

pH 5.0 yielded a k_{cat} value of 2.5 sec^{-1} for this reaction, and on the assumption that k_{cat} reaches a maximum value when an ionizing group of the enzyme with pK(app) 7.0 is completely ionized (for a review of this subject, see Reference 5), k_{cat} is expected to have a value of 0.03 sec^{-1} at pH 3.1 (see Table I). This is in agreement with a value of 0.03 sec^{-1} calculated from the rate constants k_{23} and k_{34} for k_{cat} of the Ac-Phe-OMe hydrolysis.

Pre-Steady State Kinetic Investigations of α-Chymotrypsin-catalyzed Hydrolysis of Ac-Trp-ONp—This reaction was in-

TABLE II

Rate and equilibrium constants pertaining to α-chymotrypsin-catalyzed hydrolysis of Ac-Trp-ONp

Stopped flow measurements were made at 26.5 ± 0.5° and steady state kinetic measurements at 25.0°. E_0, S_0, and F_0 refer to initial concentrations of enzyme, substrate and proflavin. All solutions contained 1.6% acetonitrile and had an ionic strength of 0.05. The solutions at pH levels 3.5 and 4.3 were buffered with potassium acetate.

pH	Stopped flow measurements					Steady state measurements: k_{cat}/K_m (app) $\times 10^{-5}$
	Concentrations	Observed release	k_{23}	$K'_S \times 10^5$	k_{23}/K'_S[a] $\times 10^{-5}$	
	μM		sec^{-1}	M	$M^{-1}\ sec^{-1}$	$M^{-1}\ sec^{-1}$
2.5	$E_0 = 4$ or 8 $S_0 = 25$–100 $F_0 = 50$	Proflavin			0.07 ± 0.1	
	$E_0 = 29$–132 $S_0 = 2.0$	*p*-Nitrophenol	3.9 ± 0.4[b]	39 ± 4[b]	0.10 ± 0.1	
3.5	$E_0 = 5$ $S_0 = 25$–100 $F_0 = 50$	*p*-Nitrophenol Proflavin			1.2 ± 0.1 1.1 ± 0.1	
	$E_0 = 5$ $S_0 = 25$–100	*p*-Nitrophenol			1.0 ± 0.1	1.0
	$E_0 = 15$–180 $S_0 = 2$	*p*-Nitrophenol	24 ± 2[b]	18 ± 1[b]	1.3 ± 0.1	
4.3	$E_0 = 5$ $S_0 = 25$–100 $F_0 = 50$	*p*-Nitrophenol Proflavin			9 ± 1 10 ± 1	

[a] In presence of proflavin, the values reported are $k_{23}/K'_S\,[1 + (F_0/K_{EF})]$.

[b] These values are not considered valid. Apparent Michaelis-Menten kinetics is thought to arise due to a nonlinear relationship between k_{obs} and enzyme concentration at constant S_0 (31) (see text).

vestigated by stopped flow techniques under several conditions of pH and of substrate, enzyme, and proflavin concentrations, and with measurements of *p*-nitrophenol (P_1) or proflavin release. Fig. 3 shows data obtained at pH 3.5 and pH 4.3. Results are summarized in Table II. The experimental conditions used were as follows. Condition I(A): $S_0 \gg E_0 < F_0$, with measurements of *p*-nitrophenol release (● in Fig. 3); Condition I(B): $S_0 \gg E_0$, with measurement of *p*-nitrophenol release (○ in Fig. 3); Condition II: $S_0 \gg E_0 < F_0$, with measurement of proflavin release (▲ in Fig. 3); Condition III: $E_0 \gg S_0$, with measurement of *p*-nitrophenol release (□ in Fig. 3). Equations relating to these experimental conditions are given under "Appendix."

Because of the limited solubility of the substrate, a very great excess of S_0 over E_0 could not, however, be attained (see Table II). In those experiments with excess substrate (○, ●, and ▲ in Fig. 3), the ($k_{obs} - k_{34}$) values define a line which is parallel to the *ordinate*; that is, they are directly proportional to S_0. In view of the relative magnitudes of K'_S and S_0, the exhibition of apparent second order kinetics is not surprising: the few values of K'_S that have been measured (8–10) for chymotrypsin and specific ester or amide substrates are of the order of 1 mM, but it was not possible to obtain S_0 in concentrations greater than 0.1 mM. As a consequence of this apparent second order kinetics, only the ratio k_{23}/K'_S—or, in the presence of proflavin, $k_{23}/K'_S[1 + (F_0/K_{EF})]$—could be obtained in these experiments. These ratios were obtained from the Fig. 3 plots as intercepts of the *abscissa* (see Equations I-7 and II-7 under "Appendix"); each k_{23}/K'_S value listed in Table II represents the average of all of the $(k_{obs} - k_{34})/S_0$ values obtained in a given experiment.

When $E_0 \gg S_0$ (□ in Fig. 3), apparent Michaelis-Menten kinetics are obtained in a given experiment. The *solid line* through □ in Fig. 3*a* was computed by the method of least squares on the basis of Michaelis-Menten kinetics (Equation III-6). Values of k_{23} and K'_S calculated on the assumption of Michaelis-Menten kinetics are listed in Table II for pH levels 2.5 and 3.5. It may be noted that in this pH region k_{23} increases with pH by a factor of about 6. For assessment of these data with $E_0 \gg S_0$, see "Discussion".

It can be seen in Fig. 3 and Table II that in the presence of proflavin there is good agreement between the data obtained by measurement of proflavin release (▲) and by measurement of *p*-nitrophenol release (●). In this connection it may be noted that, under identical experimental conditions of $S_0 \gg E_0 < F_0$, the calculation of k_{obs} depends on two different equations: Equation I-9 when *p*-nitrophenol release is measured, and Equation II-9 when proflavin displacement is measured.

It may be seen from the data in Table II that k_{23}/K'_S for experiments without proflavin and $k_{23}/K'_S[1 + (F_0/K_{EF})]$ for experiments with proflavin have about the same value at pH 2.5 and pH 3.5. This is not surprising, because of the value of the proflavin-chymotrypsin dissociation constant, K_{EF}. Determination of this constant by a method previously described (10) yielded the value $K_{EF} = 0.2$ mM at pH 3.5 (determined at 20°, with solutions of ionic strength 0.05, containing 1.6% acetonitrile). Therefore, at the concentration of proflavin used ($F_0 =$ 50 μM), K'_S is expected to have nearly the same value in the

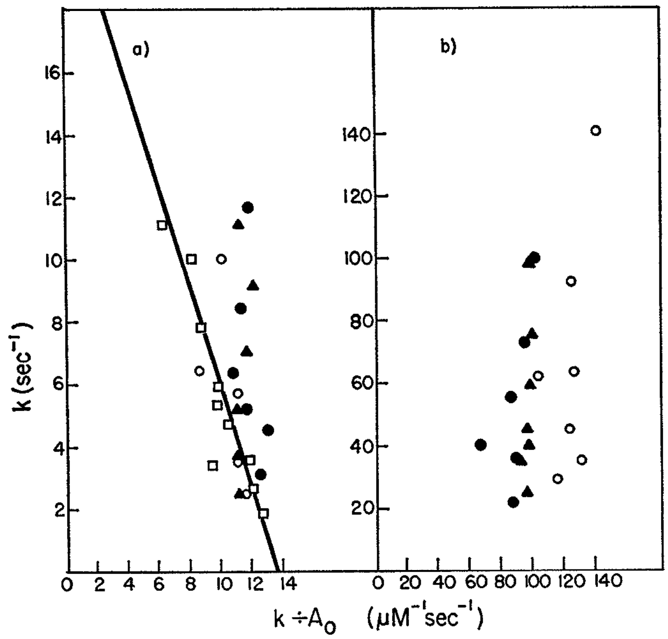

FIG. 3. The α-chymotrypsin-catalyzed hydrolysis of Ac-Trp-ONp as measured by the stopped flow technique at 26.5° at (*a*) pH 3.5 and (*b*) pH 4.3. Measurements were of the time-dependent change in concentration of the enzyme-proflavin complex as followed at 480 mμ or the *p*-nitrophenol release as followed at 348 mμ. Experimental conditions (see "Experimental Procedure") are represented as: ●, $S_0 \gg E_0 < F_0$, with measurement of *p*-nitrophenol release; ○, $S_0 \gg E_0$, with measurement of *p*-nitrophenol release; ▲, $S_0 \gg E_0 < F_0$, with measurement of proflavin release; □, $E_0 \gg S_0$, with measurement of *p*-nitrophenol release (the *line* in *a* is drawn through these points). For experiments in which $S_0 \gg E_0$, k represents ($k_{obs} - k_{34}$) and A_0 is S_0; for experiments in which $E_0 \gg S_0$, k represents k_{obs} and A_0 is E_0. Solutions contained 0.05 M potassium acetate buffer and 1.6% acetonitrile, and had ionic strength of 0.05.

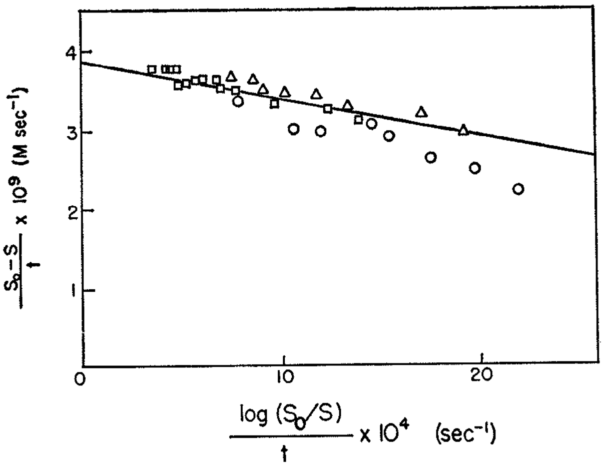

FIG. 4. Steady state kinetic measurement of the α-chymotrypsin-catalyzed hydrolysis Ac-Trp-ONp at pH 3.5 and 25.0°, plotted in accordance with Equation 5. Measurements of *p*-nitrophenol release as a function of time were made in the Cary spectrophotometer at 320 mμ. Initial enzyme concentration was 0.2 μM, and the solutions were 0.1 M in potassium acetate and contained 1.6% acetonitrile. Initial substrate concentrations were: □, 4 μM; △, 2.5 μM; ○, 1.5 μM. The *line* was established by a least squares computer program. Similar results were obtained from measurements made in presence of 50 μM proflavin.

TABLE III

Rate and equilibrium constants pertaining to α-chymotrypsin-catalyzed hydrolysis of Fa-Tyr-OEt

All solutions contained 3% acetonitrile and had an ionic strength of 0.1. Buffers used were potassium acetate for pH 5.0 solutions and potassium phosphate for pH 6.6 solutions.

pH	k_{23}	K'_S	$k_{23}/K'_S \times 10^{-6}$	k_{cat}[a]	K_m (app)[a]
	sec^{-1}	mM	$M^{-1}\ sec^{-1}$	sec^{-1}	μM
5.0	53 ± 19[b]	0.7[b]	0.08[c] 0.05[d]	1.5	28
6.6	1400[e]	1.3[f]	0.93[d]	39	42

[a] Determined from steady state measurements at 25.0°. At pH 6.6, the experiments were also performed in absence of acetonitrile and the values obtained were within the experimental error of the values reported.

[b] Determined by stopped flow proflavin displacement method at 24.5 ± 0.5°. Values were obtained from plots of ($k_{obs} - k_{34}$) against ($k_{obs} - k_{34}$)/S_0, with k_{34} taken as equivalent to k_{cat}. The K'_S value was corrected for the presence of proflavin (47.5 μM) by dividing K'_S observed (the slope) by $[1 + (F_0/K_{EF})]$, with use of a K_{EF} value of 40 μM, determined in a separate experiment by a previously described method (10).

[c] Determined from stopped flow experiments in presence of 47.5 μM proflavin.

[d] Determined as k_{cat}/K_m (app), from spectrophotometric steady state measurements at 325 mμ and 25°.

[e] Calculated from the data obtained in stopped flow experiments at pH 5.0 and in steady state kinetic measurements at pH 5.0 and pH 6.6, with the assumption that k_{23} increases with increasing pH in the same manner as k_{cat}.

[f] Calculated from k_{23} (see Footnote *e* above) and steady state kinetic parameters according to the relation (Equation 4): k_{cat}/K_m (app) $= k_{23}/K'_S$.

presence and in the absence of proflavin (see Equation II-7 in the "Appendix").

For the sake of comparison with the measured k_{23}/K'_S value, the steady state parameters k_{cat} and K_m (app) were obtained for the α-chymotrypsin-catalyzed hydrolysis of Ac-Trp-ONp at pH 3.5. Data on the release of *p*-nitrophenol as a function of time were plotted (Fig. 4) according to the integrated Michaelis-Menten rate equation:

$$\frac{S_0 - S}{t} = k_{cat}E_0 - \frac{2.3[K_m\ (app)]\,[\log\ (S_0/S)]}{t} \tag{6}$$

and k_{cat} and K_m (app) were determined from the slope and intercept of a line drawn, according to a least squares computer program, from all of the points obtained in the experiments conducted at several S_0 concentrations. The quotient of these parameters, k_{cat}/K_m (app), is listed in Table II. According to Equation 4, this quotient should be the same as k_{23}/K'_S; the values do agree within experimental error (see Table II). The k_{cat} value obtained from the measurements of the Ac-Trp-ONp hydrolysis shown in Fig. 4 is 0.02 sec^{-1} at pH 3.5 and 25°, a value which is much smaller than the corresponding observed rate constant (k_{obs}) for attainment of steady state. This circumstance allows one to equate k_{cat} with k_{34}, as has been done in Fig. 3 and Table II.

Pre-Steady State Rate Constants for α-Chymotrypsin-catalyzed

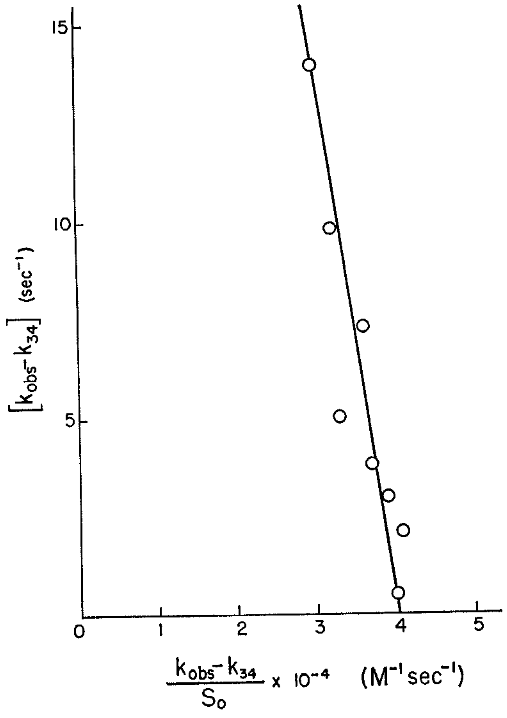

FIG. 5. Stopped flow measurement of the α-chymotrypsin-catalyzed hydrolysis of Fa-Tyr-OEt in presence of proflavin at pH 5.0 and 24.5°. Measurements were made at 480 mμ. Initial concentrations were 305 to 51 μM Fa-Tyr-OEt, 12.5 μM α-chymotrypsin, 47.5 μM proflavin, 3% acetonitrile, and 0.1 M potassium acetate.

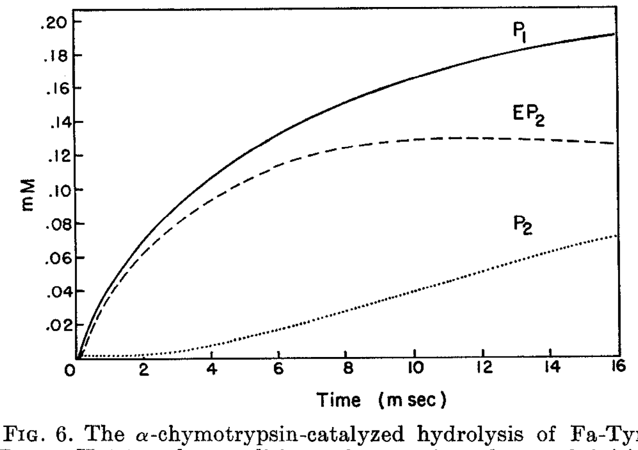

FIG. 6. The α-chymotrypsin-catalyzed hydrolysis of Fa-Tyr-OEt at pH 6.6 under conditions of approximately equal initial concentrations of enzyme and substrate. E_0 = 0.27 mM, S_0 = 0.22 mM. The *curves* were computed by means of an analogue computer and are based on experimentally obtained k_{23}, k_{34}, and K'_S values (listed in Table III) obtained under the conditions $S_0 > E_0$, conditions which allow the unambiguous evaluation of rate constants for the mechanism shown in Equation 1.

Hydrolysis of Fa-Tyr-OEt—Evaluation of the constants k_{23} and K'_S for this reaction at pH 5.0 and pH 6.6 and at 24.5 ± 0.5° is given in Table III.

The constants at pH 5.0 were evaluated from stopped flow measurements of the reaction in the presence of proflavin and with excess substrate concentration, as discussed under "Experimental Procedure." The data were plotted as (k_{obs} − k_{34}) against (k_{obs} − k_{34})/S_0 (see Equation II-7 under "Appendix"), as shown in Fig. 5. In the case of this substrate, k_{23} is sufficiently greater than k_{34} so that k_{34} could be taken as equal to k_{cat}, which was evaluated from steady state kinetic measurements.

At pH 6.6, the reaction is too fast to permit direct evaluation of k_{23} and K'_S from stopped flow measurements, but these constants could be calculated from data obtained in steady state kinetic experiments. There is a considerable amount of evidence (5, 6–10) that k_{cat} in the chymotrypsin-catalyzed hydrolysis of specific substrate esters and amides reflects k_{34} and k_{23}, respectively (see Equation 1), and that both of these rate constants are controlled by an ionizing group of the enzyme with pK(app) ~7 (5). The k_{23} value for the Fa-Tyr-OEt reaction at pH 6.6 was calculated from its value at pH 5.0 (given in Table III), on the assumption that k_{23} increases with increasing pH in the same manner that k_{cat} does; accordingly, the value obtained from stopped flow experiments at pH 5.0 was multiplied by the ratio: k_{cat} (pH 6.6) divided by k_{cat} (pH 5.0). The K'_S value was obtained from this k_{23} value, together with the ratio k_{cat}/K_m (app) obtained from steady state kinetic experiments at the same pH, for according to Equation 4 and the mechanism shown in Equation 1, k_{cat}/K_m (app) should be the same as k_{23}/K'_S.

α-Chymotrypsin-catalyzed Hydrolysis of Fa-Tyr-OEt at pH 6.6 with $E_0 \simeq S_0$—Progress curves showing the formation of P_1 (ethanol), P_2 (furylacyloyl-L-tyrosine), and EP_2 in this reaction at pH 6.6 were calculated under the conditions E_0 = 0.27 mM and S_0 = 0.22 mM. This was done by analogue computer with use of k_{23}, k_{34} (taken to be equivalent to k_{cat}), and K'_S values which were obtained under conditions $S_0 \gg E_0 < F_0$ (see Table III). These calculated curves are shown in Fig. 6. Curves representing P_1 and EP_2 formation were drawn on the basis of the computed progress curves and are shown in Fig. 7 (*triangles*). As can be seen from the data in Table III, the parameters which were used for computing the progress curves in Figs. 6 and 7 are based on both pre-steady state kinetic measurements and steady

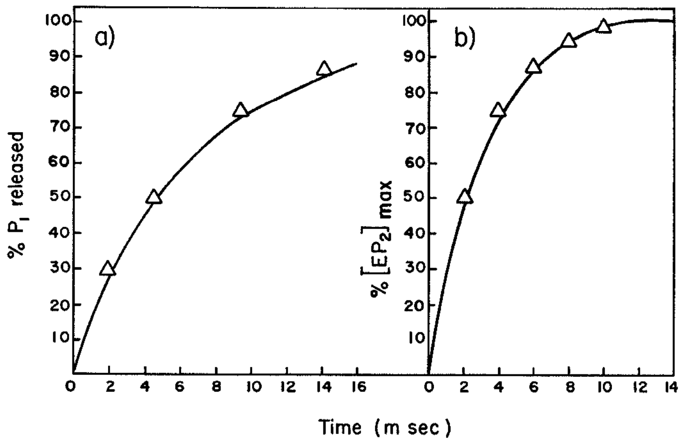

FIG. 7. A comparison of computed progress curves (see Fig. 6) with experimental points for the α-chymotrypsin-catalyzed hydrolysis of Fa-Tyr-OEt at pH 6.6 under the conditions E_0 = 0.27 mM and S_0 = 0.22 mM. Designations of P_1 and EP_2 are in accordance with the mechanism shown in Equation 1. *a*, percentage of P_1 released; *b*, percentage of maximum production of EP_2. The *curves* were drawn with use of the following experimental values obtained by Barman and Gutfreund (13) in experiments performed under the conditions $E_0 \cong S_0$: k for P_1 release = 147 sec^{-1} and $t_{\frac{1}{2}}$ for EP_2 production = 2 msec. The *open triangles* in the figure represent values computed by us (see Fig. 6) for the conditions $S_0 \cong E_0$, with use of kinetic parameters measured under the conditions $S_0 > E_0$ (see text).

state kinetic experiments. Even though the values are not known with great precision, good agreement is obtained between the measurements made by the two different methods. This is illustrated by a comparison of values for k_{23}/K'_S, obtained in flow experiments, and for k_{cat}/K_m (app) obtained from steady state kinetic experiments: values of $k_{23}/K'_S = 0.08 \times 10^6$ M^{-1} sec^{-1} and k_{cat}/K_m (app) $= 0.05 \times 10^6$ M^{-1} sec^{-1} were measured for the α-chymotrypsin-catalyzed hydrolysis of Fa-Tyr-OEt at pH 5.0 (see Table III).

A comparison of these data with corresponding experimental data (measured with $E_0 = 0.27$ mM and $S_0 = 0.22$ mM by Barman and Gutfreund) is shown in Fig. 7, in which the *solid lines* represent the experimental measurements and the *triangles* the computed values. In preparing the curves representing the measurements of Barman and Gutfreund, we used their experimentally observed rate constant for liberation of ethanol (P_1 in Equation 1) and their experimentally observed $t_{\frac{1}{2}}$ value for the formation of EP_2. (The authors estimate that because of instrumental limitations the actual $t_{\frac{1}{2}}$ value may be about 20% lower; this would not materially change the conclusions reached here.)

DISCUSSION

The data indicate that the proflavin displacement method is suitable for investigations of the kinetics of chymotrypsin-catalyzed reactions for, in the reactions investigated, the same information was obtained by the proflavin displacement method as by other independent measurements. This may be seen in Fig. 2 and Table I, which present results of experiments in which k_{23}, k_{34}, and K'_S (see Equation 1) for the chymotrypsin-catalyzed hydrolysis of Ac-Phe-OMe at pH 3.1 were determined by two independent methods. The comparison was made at low pH because in the low pH region the rates of reaction are sufficiently slow to allow accurate measurement of the small absorbance changes that accompany the reaction. The parameters obtained by these two independent methods, the proflavin displacement method and the measurement of spectral changes of the enzyme at 290 mμ, were found to agree satisfactorily. The validity of using the proflavin displacement method for measuring kinetic parameters in chymotrypsin-catalyzed reactions is also indicated by the data shown in Fig. 3 and Table II. In these experiments, both p-nitrophenol release and proflavin displacement (which indicates the formation of EP_2 in Equation 1) were measured during the chymotrypsin-catalyzed hydrolysis of Ac-Trp-ONp under the conditions $S_0 \gg E_0 < F_0$ at pH levels 2.5, 3.5, and 4.3. While substrate concentrations sufficiently high for the determination of k_{23} and K'_S separately could not be obtained, the quotient k_{23}/K'_S could be evaluated. The observation that, under the conditions $S_0 \gg E_0$ with proflavin present in the reaction solution, the k_{23}/K'_S values were the same whether p-nitrophenol release or proflavin displacement was measured is a strong indication that the same process is measured in each case.

As a check on k_{23}/K'_S values determined in stopped flow experiments such as those with Ac-Trp-ONp, the quotient of the steady state kinetic parameters, k_{cat}/K_m (app), was determined both in presence and absence of proflavin, and compared (see Table II) with the corresponding evaluation of k_{23}/K'_S. According to Equation 4, these two quotients should agree, and the values obtained did show satisfactory agreement.

Although the same results are to be expected in kinetic measurements of chymotrypsin-catalyzed hydrolysis under the experimental conditions $S_0 \gg E_0$ and $E_0 \gg S_0$, the stopped flow measurements of the Ac-Trp-ONp hydrolysis showed an apparent difference (see Fig. 3*a*). With $E_0 \gg S_0$, the reaction appeared to follow Michaelis-Menten kinetics rather than the apparent bimolecular kinetics observed when $S_0 \gg E_0$. The discrepancy between the two sets of data is most probably due to aggregation of chymotrypsin at high enzyme concentrations. The aggregation of chymotrypsin has been investigated in many laboratories (for a review of the papers, see References 28 to 30), and most recently by Kegeles *et al.* (28, 29). Although it has been concluded that the dimer and trimer bind substrate and that the dimer and trimer bind inhibitor as well as the monomer does (28, 29), the evidence is that highly polymerized species of chymotrypsin have diminished catalytic properties (30). The data for the conditions $E_0 \gg S_0$ could be fitted to a curve rather than to a straight line (*solid line* in Fig. 3*a*). In the event of enzyme aggregation, results obtained at low enzyme concentrations for experiments in which $E_0 \gg S_0$ would be expected to be the same as results for experiments in which $S_0 \gg E_0$, and this expectation is realized. As can be seen from the data at pH 3.5 in Table II, similar values for k_{23}/K'_S (the intercept of the *abscissa* in Fig. 3*a*) are obtained when $E_0 \gg S_0$ and when $S_0 \gg E_0$.

The agreement between the k_{23}/K'_S values obtained by observing either proflavin displacement or p-nitrophenol release in the experiments with Ac-Trp-ONp leads to the conclusion that, in the chymotrypsin-catalyzed hydrolysis of at least this ester substrate, the rate constant k_{23} for formation of the steady state intermediate (identified here as EP_2) is also the rate constant for the liberation of P_1 (p-nitrophenol in the case of Ac-Trp-ONp). In other words, P_1 is liberated concomitantly with the formation of EP_2, and the proflavin displacement method gives the same results in these experiments as the more direct measurement of p-nitrophenol release.

The results obtained from these experiments are in agreement with results obtained previously. In earlier experiments (9, 18–21), a thorough investigation of the spectral changes of the enzyme near 290 mμ was made, and it was established that the magnitude of these spectral changes is an indication of the concentration of the enzyme-substrate complex. The enzyme-substrate dissociation constants, K'_S, for chymotrypsin and N-acetyl-L-phenylalaninamide and for chymotrypsin and N-acetyl-L-tryptophanamide were determined (9) with use of three independent measurements: the spectral changes of the enzyme near 290 mμ, the displacement of proflavin from complex with enzyme, and the steady state kinetic parameter, K_m (app). The same values for K'_S were obtained, within experimental error, by all three methods (9).

The present experiments with the α-chymotrypsin-catalyzed hydrolysis of Fa-Tyr-OEt are also consistent with the supposition that P_1 (Equation 1) is released concomitantly with the formation of the intermediate product EP_2. Pertinent to this finding is recent work (13) of Barman and Gutfreund, who succeeded, with their excellent quenching technique, in making direct measurements of alcohol (P_1) release in the chymotrypsin-catalyzed hydrolysis of this substrate. They also measured the absorbance change at 340 mμ due to substrate binding. The data in Figs. 6 and 7 show that there is excellent agreement between progress curves obtained from these experimental measurements of Barman and Gutfreund, and comparable progress curves computed with the use of k_{23}, k_{34}, and K'_S values which were obtained by us under the conditions $S_0 > E_0$, conditions which allow unambiguous evaluation of rate constants for the mechanism shown in Equation 1.

It should be noted that, even though the experiments reported here provide no evidence for the existence of additional intermediates on the pathway between ES and EP_2 in Equation 1, these experiments do not, of course, prove that such intermediates do not exist.

In conclusion, the data presented here are consistent with the mechanism shown in Equation 1 for the chymotrypsin-catalyzed hydrolysis of specific substrate esters, which shows alcohol (P_1) released concomitantly with the formation of the intermediate EP_2. The results also indicate that the proflavin displacement method is suitable for measuring the kinetics of the chymotrypsin-catalyzed hydrolysis of specific substrates. The determination of the rate constants k_{23} and k_{34} and the dissociation constant K'_S for a series of chymotrypsin-specific substrate esters and the pH and temperature dependence of these parameters will be reported in a subsequent publication.

APPENDIX

Equations for Evaluation of Pre-Steady State Kinetic Parameters of α-Chymotrypsin-catalyzed Hydrolysis of Specific Substrate Esters

In the equations below, E, F, S, P_1, EF, SF, and EP_2 refer to molar concentrations as defined in Equation 1 of the text and the subscript zero indicates initial analytical concentration (total concentration of the substance in all its forms). K'_S and K_{EF} are the enzyme-substrate and enzyme-proflavin dissociation constants; k_{obs} is the experimentally observed rate constant for the reaction; and K'_S, k_{23}, and k_{34} are as defined by Equation 1 of the text.

Condition I(A): $S_0 \gg E_0 < F_0$, with Measurement of P_1 Release—

$$E_0 = E + ES + EF + EP_2 \tag{I-1}$$

Also:

$$K'_S = \frac{(E)(S_0)}{ES}\,;\ K_{EF} = \frac{(E)(F_0)}{EF}$$

Therefore

$$E = \frac{K'_S}{S_0}(ES) = \frac{K_{EF}}{F_0}(EF) \tag{I-2}$$

and

$$EF = \frac{K'_S}{S_0}(ES)\frac{F_0}{K_{EF}} \quad \text{or} \quad ES = (EF)\frac{K_{EF}S_0}{F_0K'_S} \tag{I-3}$$

Substitution of Equations I-2 and I-3 into Equation I-1 yields

$$E_0 = ES\left(1 + \frac{K'_SZ}{S_0}\right) + EP_2$$

where $Z = 1 + (F_0/K_{EF})$. Therefore

$$EP_2 = E_0 - (ES)\frac{S_0 + K'_SZ}{S_0} \tag{I-4}$$

$$\frac{d(EP_2)}{dt} = k_{23}(ES) - k_{34}(EP_2)$$

Therefore:

$$\frac{d\left[E_0 - (ES)\dfrac{S_0 + K'_SZ}{S_0}\right]}{dt} = k_{23}(ES) - k_{34}\left[E_0 - (ES)\frac{S_0 + K'_SZ}{S_0}\right] \tag{I-5}$$

Integration of Equation I-5 gives the concentration of ES at time t:

$$[ES]_t = \frac{E_0S_0}{S_0(k_{23} + k_{34}) + k_{34}K'_SZ} \cdot \left[k_{34} + \left(\frac{k_{23}S_0}{S_0 + K'_SZ}\right)e^{-k_{obs}t}\right] \tag{I-6}$$

where

$$k_{obs} = \frac{k_{23}S_0}{S_0 + K'_SZ} + k_{34} \tag{I-7}$$

$$\frac{d(P_1)}{dt} = k_{23}(ES) = \frac{k_{23}E_0S_0}{S_0(k_{23} + k_{34}) + k_{34}K'_SZ} \cdot \left[k_{34} + \left(\frac{k_{23}S_0}{S_0 + K'_SZ}\right)e^{-k_{obs}t}\right] \tag{I-8}$$

Integration of Equation I-8 gives the concentration of P_1 at time t:

$$[P_1]_t = \frac{k_{23}k_{34}E_0S_0t}{S_0(k_{23} + k_{34}) + k_{34}K'_SZ} + E_0\left[\frac{k_{23}S_0}{S_0(k_{23} + k_{34}) + k_{34}K'_SZ}\right]^2(1 - e^{-k_{obs}t}) \tag{I-9}$$

When P_1 is determined as a function of time, k_{obs} can be evaluated by use of a modified Guggenheim plot, used previously by Gutfreund and Sturtevant (2). Then when, Z and k_{34} are known, Equation I-7 can be used to evaluate k_{23} and K'_S from the k_{obs} values determined at various substrate concentrations.

Condition I(B): $S_0 \gg E_0$, with Measurement of P_1 Release—The pertinent equations are those given above, without terms involving proflavin (F_0, EF, K_{EF}). Equations I-4 through I-9, with $Z = 1$, are applicable.

Condition II: $S_0 \gg E_0 < F_0$, with Measurement of Proflavin Release—Equations I-2 and I-3 (above) are applicable. Substitution of Equations I-2 and I-3 into Equation I-1 yields:

$$E_0 = EF\left(\frac{K_{EF}}{F_0} + \frac{K_{EF}}{F_0}\frac{S_0}{K'_S} + 1\right) + EP_2$$

Therefore:

$$EP_2 = E_0 - (EF)\Phi$$

where

$$\Phi = \frac{K_{EF}}{F_0} + \frac{K_{EF}S_0}{F_0K'_S} + 1$$

Then, since

$$\frac{d(EP)}{dt} = k_{23}(ES) - k_{34}(EP)$$

it follows that

$$\frac{d[E_0 - (EF)\Phi]}{dt} = k_{23}(EF)\frac{K_{EF}S_0}{F_0K'_S} - k_{34}[E_0 - (EF)\Phi] \quad \text{(II-5)}$$

Integration of Equation II-5 gives the concentration of EF at time t:

$$[EF]_t = \frac{E_0F_0}{S_0(k_{23} + k_{34}) + k_{34}K'_SZ}\left(\frac{K'_S}{K_{EF}}\right)\left[k_{34} + \frac{k_{23}S_0}{S_0 + K'_SZ}(e^{-k_{obs}t})\right] \quad \text{(II-6)}$$

where

$$k_{obs} = \frac{k_{23}S_0}{S_0 + K'_SZ} + k_{34} \quad \text{(II-7)}$$

The steady state concentration of EF is obtained from a combination of Equations I-1, I-2, and I-3, and the relation $k_{23}(ES) = k_{34}(EP_2)$.

$$[EF]_{ss} = \frac{k_{34}E_0F_0}{S_0(k_{23} + k_{34}) + k_{34}K'_SZ}\left(\frac{K'_S}{K_{EF}}\right) \quad \text{(II-8)}$$

Then

$$[EF]_t - [EF]_{ss} = \frac{1}{\epsilon_M^{EF}}[(A_{465})_t - (A_{465})_{ss}] = \frac{k_{23}E_0S_0}{S_0 + K'_SZ}\left\{\frac{K'_SF_0}{K_{EF}[S_0(k_{23} + k_{34}) + k_{34}K'_SZ]}\right\}e^{-k_{obs}t} \quad \text{(II-9)}$$

Here $(A_{465})_t$ is the absorbance of the solution in the stopped flow apparatus at 465 mμ at a time t, and $(A_{465})_{ss}$ is the absorbance when a steady state concentration of material absorbing at 465 mμ (F and EF) has been reached (see Fig. 1). The k_{obs} value can be evaluated from

$$\ln[(A_{465})_t - (A_{465})_{ss}] = C - k_{obs}t \quad \text{(II-10)}$$

With such determinations of k_{obs} at various substrate concentrations, k_{23} and K'_S can be evaluated according to Equation II-7, provided that Z and k_{34} are known.

Condition III: $E_0 \gg S_0$, with Measurement of P_1 Release—

$$S_0 = S + ES + P_1 \quad \text{(III-1)}$$

$$K'_S = \frac{(E_0)(S)}{ES}; \text{ or } S = \frac{K'_S}{E_0}(ES) \quad \text{(III-2)}$$

Substitution of Equation III-2 into Equation III-1 yields

$$S_0 = ES\left(\frac{K'_S + E_0}{E_0}\right) + P_1$$

Therefore

$$ES = \frac{E_0S_0}{K'_S + E_0} - \frac{P_1E_0}{K'_S + E_0} \quad \text{(III-3)}$$

$$\frac{d(P_1)}{dt} = k_{23}(ES) = k_{23}\left[\frac{E_0S_0}{K'_S + E_0} - P_1\frac{E_0}{K'_S + E_0}\right] \quad \text{(III-4)}$$

Integration of Equation III-4 gives the concentration of P_1 at time t:

$$[P_1]_t = S_0(1 - e^{-k_{obs}t}) \quad \text{(III-5)}$$

where

$$k_{obs} = \frac{k_{23}E_0}{K'_S + E_0} \quad \text{(III-6)}$$

When P_1 is determined as a function of time, k_{obs} can be evaluated from

$$\ln\frac{S_0 - [P_1]_t}{S_0} = -k_{obs}t \quad \text{(III-7)}$$

Then, with the determination of k at various initial enzyme concentrations, k_{23} and K'_S can be determined by the use of Equation III-6.

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