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Studies of the activity of chymotrypsin

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Physical and chemical studies of chymotrypsin in solution and kinetic measurements of individual steps in reactions catalysed by this hydrolytic enzyme are reported. The solution studies also provide a basis for correlation of individual reaction steps with structural information obtained by Drs Blow and Hartley and discussed at this Discussion.

The rate and equilibrium constants pertaining to the three-step mechanism shown below have been determined for the chymotrypsin catalysed hydrolysis of three specific ester substrates—the ethyl esters of *N*-acetyl-L-tyrosine, *N*-acetyl-L-tryptophan, and *N*-acetyl-L-phenylalanine—at selected pH values with use of both flow and relaxation techniques.

$$E + S \underset{}{\overset{K'_S}{\rightleftharpoons}} ES \overset{k_{23}}{\rightarrow} EP_2 \overset{k_{34}}{\longrightarrow} E + P_2 \quad (P_1 \text{ released at } ES \rightarrow EP_2)$$

where E is enzyme, S is substrate, ES is a complex, EP_2 is an intermediate compound, and the products P_1 and P_2 are, respectively, an alcohol or amine and an acid.

The hydrolysis of chymotrypsin specific substrates is shown to be considerably more complex than is indicated by this previously proposed mechanism. In addition to the steps shown, there exist a number of ionization and conformational equilibria which account for the bell-shaped pH–rate profiles observed for chymotrypsin catalysed reactions. A catalytically active enzyme conformation, which is in pH dependent equilibrium with a catalytically inactive conformation, is shown to have its origin in the enzyme activation mechanism. In the chymotrypsin catalysed hydrolysis of an amide substrate, furylacryloyltryptophanamide, there has been detected an additional intermediate which differs from the intermediate EP_2 observed in ester hydrolysis.

1. Introduction

The activity of chymotrypsin in solution will be discussed in this paper. To be considered in particular is information about individual steps in chymotrypsin catalysed reactions that we have obtained from chemical and physical studies of the enzyme in solution. Since there are conformational changes of the enzyme involved in the catalytic reactions, there will be an opportunity to correlate some of our studies with the elegant structural work already discussed by Birktoft *et al.* (p. 67) and by Hartley (p. 77).

The function of chymotrypsin is the catalytic hydrolysis of proteins in the intestine. Cleavage of amide bonds occurs adjacent to the carboxyl function of the aromatic amino acid residues—phenylalanine, tyrosine, and tryptophan. The hydrolysis of these proteins occurs faster by a factor of about 10^5 in the presence of proteolytic enzymes than in their absence; digestive processes involving proteins would therefore take about 50 years in the absence of these enzymes.

The three-step mechanism that has been proposed for chymotrypsin catalysed catalysis is

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \overset{k_{23}}{\rightarrow} EP_2 \overset{k_{34}}{\longrightarrow} E + P_2 \quad (P_1 \text{ released at } ES \rightarrow EP_2) \qquad (1)$$

where E is enzyme, S is substrate, ES is a complex, EP_2 is an enzyme–substrate compound whose formation involves the hydroxyl group of Ser-195 in the active site of chymotrypsin, and P_1 and P_2 are the products of the catalytic hydrolysis. P_1 is either an alcohol or an amine, depending on whether the substrate is an ester or an amide; P_2 is an acid. K_S is an enzyme–substrate dissociation constant, and k_{23} and k_{34} are the rate constants pertaining to the liberation

of P_1 and P_2, respectively. This mechanism is based on investigations by Hartley & Kilby (1954), Gutfreund & Sturtevant (1956), and Faller & Sturtevant (1966) of the hydrolysis of the model substrate *p*-nitrophenyl acetate. The applicability of this mechanism to the chymotrypsin catalysed hydrolysis of specific substrate esters was suggested by subsequent steady state kinetic investigations by Niemann (1964) and by Bender & Kézdy (1965).

Steady state kinetic studies, however, yield only combinations of rate and equilibrium constants. The relations between the parameters pertaining to the relatively simple mechanism shown in equation (1) and the measurable steady state kinetic parameters (the catalytic rate constant, k_{cat}, and the Michaelis–Menten constant, K_m) may be expressed (Gutfreund & Sturtevant 1956):

$$k_{cat} = k_{23}\, k_{34}/(k_{23}+k_{34}) \quad (2)$$

$$K_m = K_S\, k_{34}/(k_{23}+k_{34}). \quad (3)$$

It will be shown that chymotrypsin catalysed reactions are considerably more complex than is indicated by the mechanism given in equation (1), and that both the specificity and efficiency of a chymotrypsin catalysed reaction depends on the interaction of many processes involving a great number of rate constants and ionization constants. In order to understand this complex reaction, it will be necessary to know all the steps in the reaction and their sequence along the reaction path. This has invariably been found to be true for even very simple reactions. We chose to approach the problem of elucidating individual steps in chymotrypsin catalysed reactions by studying physical changes of the enzyme that accompany these reactions. This approach not only makes possible the measurement of the kinetics of the intermediate steps, but also provides a basis for correlation of these individual steps with information obtainable from structural studies.

Some of the physical changes of the enzyme which we have observed in studies of chymotrypsin catalysed reactions (Wootton & Hess 1962; Havsteen & Hess 1963; Moon, Sturtevant & Hess 1965; Hess 1968; McConn, Fasman & Hess 1969), and which have been useful in obtaining structural as well as kinetic information, are described as follows: (1) The interaction of enzyme with both specific inhibitors and specific substrates is accompanied by spectral changes of the enzyme at 290 nm. (2) The specific rotation and circular dichroism spectra of the free enzyme are pH dependent; changes in these measurements are observed as a result of substrate binding above neutral pH. (3) The enzyme takes up hydrogen ions from solution when it binds substrate above neutral pH.

2. Conformation of chymotrypsin and its relation to the pH dependence of chymotrypsin catalysed reactions

Chymotrypsin catalysed reactions have bell-shaped pH–rate profiles (Bender & Kézdy 1965), such as the one shown in figure 1 for the hydrolysis of a neutral substrate, acetyl-L-tryptophanamide. The bell-shaped pH–rate profile has invariably been interpreted as evidence that two ionizing groups of the enzyme are important in the catalytic reaction. The mid-point of the left limb of the pH–rate profile illustrated indicates that the rate of the catalytic reaction increases with the ionization of an enzymic group with $pK_{app} \sim 7.0$. This ionizing group has been identified from chemical experiments (Weil, James & Buchert 1953; Ray & Koshland 1960; Schoellmann & Shaw 1963; Hartley 1964) as the imidazole nitrogen of His-57. The mid-point

of the right side of the pH–rate profile indicates that the rate of the catalytic reaction decreases as an ionizing group of the enzyme with $pK_{app} \sim 8.5$ ionizes. This ionizing group has been identified by Labouesse, Oppenheimer & Hess (1964) and Oppenheimer, Labouesse & Hess (1966) as the α-amino group of Ile-16.

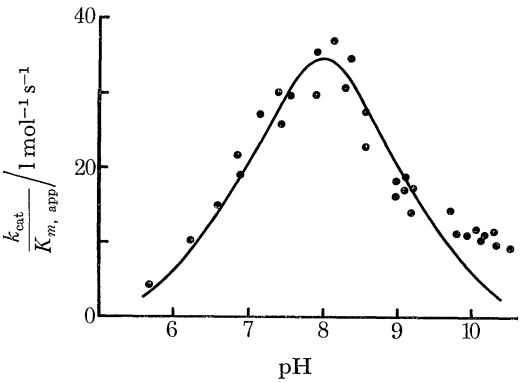

FIGURE 1. A pH–rate profile typical of reactions catalysed by chymotrypsin. This curve was drawn from steady state kinetic data on the δ-chymotrypsin catalysed hydrolysis of *N*-acetyl-L-tryptophanamide at 25 °C. Continuous measurements of released ammonia were made at each pH level by means of a modified Technicon amino acid analyser over a range of substrate concentrations. Eadie plots yielded values of k_{cat} and $K_{m,app}$. These measurements were made at an initial enzyme concentration of from 1 to 6 μmol l^{-1} and substrate concentration in the range 2 to 20 mmol l^{-1}. Appropriately buffered solutions also contained KCl to bring the ionic strength to 0.16 mol l^{-1}.

(*a*) *The* pH *dependence of the catalytic reactions at alkaline* pH

The data reported here indicate that the pH dependence at alkaline pH of chymotrypsin catalysed reactions is related to the events that accompany the conversion of catalytically inactive chymotrypsinogen to the enzyme (Labouesse *et al.* 1964; Oppenheimer *et al.* 1966). Studies of Neurath and Desnuelle and their associates (Davie & Neurath 1955; Dreyer & Neurath 1955; Rovery, Poilroux & Desnuelle 1955; Desnuelle & Fabre 1955) showed that the one critical chemical event in the activation of chymotrypsinogen is the splitting of a single peptide bond by the enzyme trypsin to produce a new N-terminal residue, that of Ile-16 (see figure 2). Neurath, Rupley & Dreyer (1956) further suggested that the activation is accompanied by a conformational change which establishes the active site of the enzyme. Evidence that the conformations of chymotrypsinogen and chymotrypsin are different at neutral pH has been presented (Imahori, Yoshida & Hasizume 1960; Raval & Schellman 1965; Biltonen, Lumry, Madison & Parker 1965*a*, *b*; Fasman, Foster & Beychok 1966). Evidence of this conformational difference is also provided by the difference between the circular dichroism spectra of chymotrypsinogen and chymotrypsin at neutral pH, apparent from the data in figure 2. The circular dichroism data shown in figure 2 are similar to those obtained previously by Fasman *et al.* (1966).

The relation between conformational forms of chymotrypsin and the ionization state of the α-amino group of Ile-16 is demonstrated in figures 3 to 6. Titration difference curves (Moon *et al.* 1965; Oppenheimer *et al.* 1966; Havsteen & Hess 1964), which serve to identify the group and demonstrate its ionization characteristics in chymotrypsin and related compounds, are shown in figure 3. The solid line represents the difference in titratable groups between δ-chymotrypsin and chymotrypsinogen. This titration difference curve indicates that δ-chymotrypsin

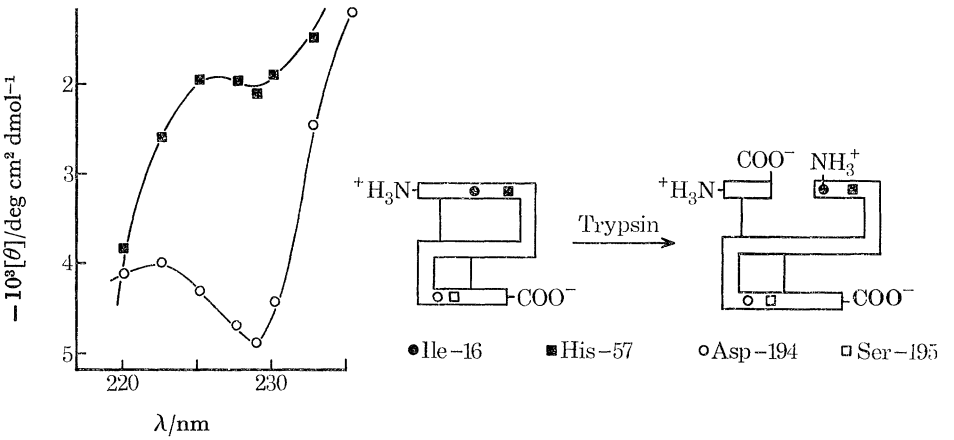

FIGURE 2. Conformational changes of the protein molecule that occur when chymotrypsinogen is activated at neutral pH. The diagram illustrates the breaking of the peptide chain that occurs when chymotrypsinogen is activated by trypsin to form chymotrypsin: the α-amino group of Ile-16 is released from peptide linkage and becomes free to ionize. The catalytic site of the enzyme includes Asp-194 and Ser-195. The circular dichroism spectra of ■, chymotrypsinogen, and ○, α-chymotrypsin, at pH 6.7 and room temperature (23 °C) are compared in the plot of ellipticity against wavelength. Measurements were made on a Cary 60 recording spectropolarimeter equipped with a 6001 circular dichroism attachment, and values of ellipticity, θ, were calculated as previously described by Fasman *et al.* (1966). Appropriately buffered solutions contained 0.04 mmol l^{-1} of protein and 0.1 mol l^{-1} of Na_2SO_4 to give an ionic strength of 0.25 mol l^{-1}.

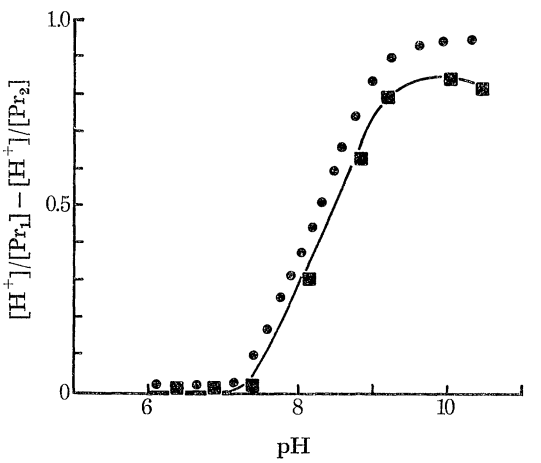

FIGURE 3. The difference in titratable groups in chymotrypsin and in related proteins in which the α-amino group of Ile-16 of the enzyme is prevented from ionizing. Plotted against pH are differences between titrated hydrogen ion for the two proteins (designated 1 and 2) that are being compared. In all instances, the proteins were prepared from chymotrypsinogen that had been acetylated. The solid line is for δ-chymotrypsin and chymotrypsinogen; ■, δ-chymotrypsin and δ-chymotrypsin in which the α-amino group of Ile-16 is blocked by acetylation; ●, δ-chymotrypsin and di-isopropylphosphoryl-chymotrypsin, an inhibition product. Measurements were made with a Radiometer pH-stat (TTT 1c) at 4 °C, with solutions containing 0.4 μmol l^{-1} of protein and 0.15 mol l^{-1} of KCl.

has an ionizing group, with a pK_{app} of about 8.5, which does not ionize in chymotrypsinogen. One group that cannot ionize in chymotrypsinogen, because it is in peptide linkage, is the α-amino group of Ile-16. The circular points give the difference in titratable groups between δ-chymotrypsin and δ-chymotrypsin in which the α-amino group of Ile-16 has been specifically acetylated and thereby prevented from ionizing. These data identify the group with pK_{app} ~ 8.5 as the N-terminal α-amino group of Ile-16 of chymotrypsin. The solid black squares

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give the difference in titratable groups between δ-chymotrypsin and di-isopropylphosphoryl-δ-chymotrypsin, an inhibition product. These measurements indicate that in the inhibited enzyme, the critical isoleucine α-amino group is prevented from ionizing.

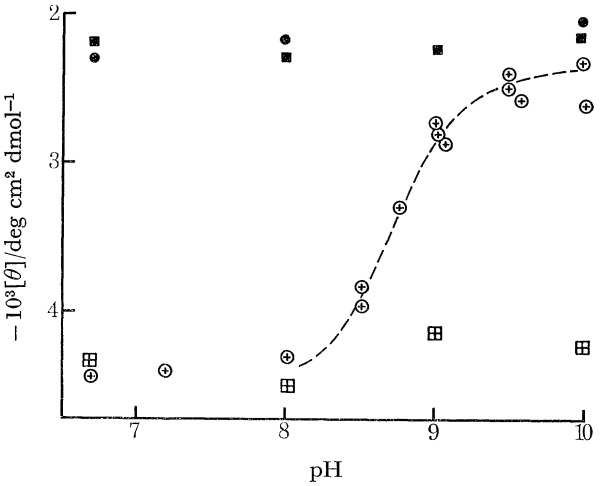

FIGURE 4. The pH dependence of circular dichroism of chymotrypsin, as compared with related, catalytically inactive proteins. Ellipticity at 230 nm (see legend of figure 2) is plotted against pH. The circles with + signs represent data for an acetylated δ-chymotrypsin. The dashed line was calculated on the assumption that a single ionizing group of $pK_{app} \sim 8.5$ controls the change in ellipticity. The solid circles and squares represent, respectively, acetylated δ-chymotrypsin in which the α-amino group of Ile-16 has also been acetylated, and acetylated chymotrypsinogen, in which this group is in peptide linkage. The open squares with + signs represent the inhibition product di-isopropylphosphoryl-chymotrypsin, in which the conformation characteristic of active enzyme has evidently been stabilized by the binding of inhibitor.

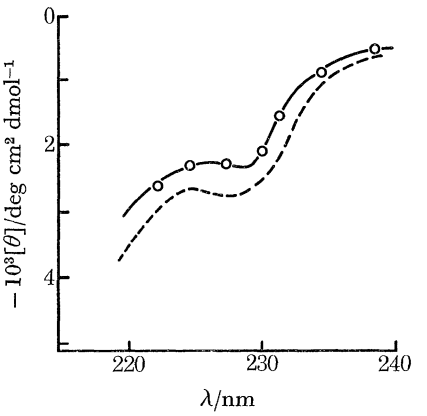

FIGURE 5. Circular dichroism spectra of chymotrypsin and related proteins in which the α-amino group of Ile-16 is blocked. The solid line represents measurements of acetylated chymotrypsinogen; the dashed line is for acetylated δ-chymotrypsin prepared by activation of the acetylated zymogen; and the circles are for acetylated δ-chymotrypsin in which the α-amino group of Ile-16 has also been acetylated. Measurements were made at pH 10 and room temperature (for experimental details, see legend of figure 2).

The alkaline pH dependencies of circular dichroism measurements of δ-chymotrypsin and three related proteins are shown in figure 4, which gives measurements of the ellipticity band centred near 230 nm. The open circles with plus signs on the dashed line pertain to δ-chymotrypsin; the line itself is calculated on the assumption that a single ionizing group of the enzyme with $pK_{app} \sim 8.5$ controls the change in the circular dichroism spectra. The solid circles

correspond to the measurements of chymotrypsin in which the α-amino group of Ile-16 has been specifically acetylated. The open squares with plus signs represent data for the inhibited enzyme, di-isopropylphosphoryl-δ-chymotrypsin, a molecule in which the hydroxyl group of serine residue 195 has been phosphorylated. At pH 10 and above, the circular dichroism measurements for chymotrypsinogen, δ-chymotrypsin, and acetylated δ-chymotrypsin are similar. The data in figure 5 show this similarity of the circular dichroism in the spectra at pH 10 for chymotrypsinogen, δ-chymotrypsin, and δ-chymotrypsin in which the α-amino group of

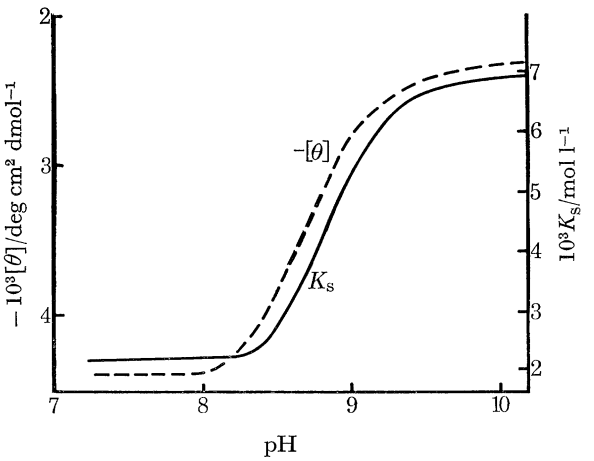

FIGURE 6. The pH dependency of circular dichroism and of K_S, the enzyme–substrate dissociation constant pertaining to δ-chymotrypsin and the neutral substrate *N*-acetyl-L-tryptophanamide. The K_S values were determined as previously described (Himoe *et al.* 1967), and values of ellipticity, θ, at $\lambda = 230$ nm, were determined as described in the legend of figure 2.

Ile-16 has been specifically acetylated. Our interpretation of these results is that molecules which carry a positive charge on Ile-16 exist in one conformational form, and molecules in which this group cannot or does not ionize exhibit another conformational form. In the three proteins that exhibit the same circular dichroism spectra at pH 10 and above (see figures 4 and 5), the α-amino group is unprotonated—in the chymotrypsinogen because it is in peptide linkage, in the δ-chymotrypsin because at high pH it is ionized, and in the acetylated δ-chymotrypsin because it is specifically blocked by acetylation. The phosphorylated inhibition product (open squares in figure 4) has a pH independent circular dichroism equivalent to that of δ-chymotrypsin at neutral pH. The inference of this finding is that in the phosphorylated enzyme, the α-amino group of Ile-16 maintains a positive charge throughout the pH region 7 to 10, a behaviour that is supported by the titration data shown in figure 3.

The data in figure 6 illustrate the relation in the alkaline pH region between circular dichroism of chymotrypsin (dashed line) and the value of K_S, the enzyme–substrate dissociation constant pertaining to chymotrypsin and a neutral substrate (solid line). The chymotrypsin specific substrate used in the experiment is *N*-acetyl-L-tryptophanamide. It is apparent from the data that K_S, like the circular dichroism of the free enzyme, depends on the ionization of a group with $pK_{app} \sim 8.5$. Kinetic experiments (Himoe & Hess 1966; Himoe, Parks & Hess 1967) with a series of substrates have, in fact, indicated that the pH dependence of chymotrypsin catalysed reactions at alkaline pH is due to the pH dependence of K_S.

The origin of the pH dependence of chymotrypsin catalysed reactions may be inferred from

the results of these titration, circular dichroism, and K_S measurements. The suggestion is that the ionization of the α-amino group of Ile-16 controls the equilibrium between two main conformations of the enzyme. This is illustrated in figure 7. Enzyme molecules in which this α-amino group carries a positive charge (referred to as conformation AH in figure 7) bind substrate better than molecules having the conformational form in which this group is uncharged (conformation I).

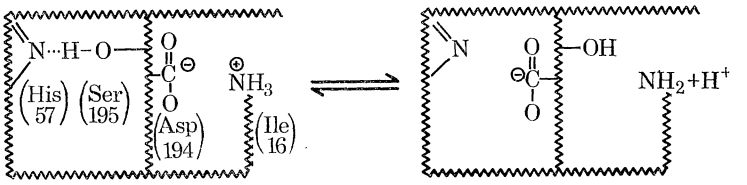

FIGURE 7. Representation of conformational forms deduced for chymotrypsin at neutral and high pH levels. In the active form (AH), the α-amino group of Ile-16 is protonated and forms an internal ion pair with the negatively charged carboxyl group of Asp-194, thereby permitting an apparently crucial spatial relation between His-57 and Ser-195 in the active site of the enzyme. In the high pH form (I), the isoleucine α-amino group has lost its charge and Asp-194, no longer held in the interior of the molecule, moves to disrupt the binding site.

Our interpretation of the pH dependence of the enzyme–substrate dissociation constant led to several predictions that we could test. If substrate binds more readily to the neutral pH conformation of the enzyme than to the conformation that is predominant at high pH, it follows that the process of substrate binding must bring about a shift in the equilibrium between conformations in the direction of the neutral-pH form (conformation AH). Such a conversion of enzyme molecules in which the α-amino group is not protonated, to a conformation in which this group is protonated, would be observable as an uptake of protons from solution by the enzyme. Similarly, when there is a difference in circular dichroism between the low and high pH conformations of chymotrypsin, substrate binding would be expected to produce changes in these parameters, since molecules having the conformation characteristic of neutral pH enzyme would be more predominant in the complexes than in the free enzyme. Both of these predictions have been realized, as is demonstrated by the data shown in figures 8 and 9.

Figure 8 shows changes in circular dichroism of chymotrypsin at 230 nm caused by the presence of varying amounts of the specific inhibitor indole. The slope of the line is proportional to $K_{m,\,app}$; the value of 0.3 mmol l^{-1} obtained from this slope is in reasonable agreement with a value of $K_{m,\,app}$ obtained in steady state kinetic experiments. The intercept of the ordinate represents the circular dichroism of enzyme saturated with inhibitor; this value is in fairly good agreement with the circular dichroism measurement at 230 nm obtained with the free enzyme at neutral pH.

The data in figure 9 show that proton uptake by chymotrypsin is observed as a result of the binding of substrate or inhibitor (McConn, Ku, Odell, Czerlinski & Hess 1968). Data are shown for the binding of the inhibitor indole to α-chymotrypsin, *N*-acetyl-D-tryptophanamide to α-chymotrypsin, and *N*-acetyl-L-tryptophanamide to δ-chymotrypsin. The values used in the figure were obtained by determining the proton uptake at each pH level as a function of substrate concentration, and extrapolating to infinite substrate concentration. It may be noticed that each of the substrates measured produced a different maximum proton uptake, indicating that the equilibrium between enzyme conformations is affected differently by different substrates.

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The data for each substrate yield two values of pK_{app}, one for the free enzyme and a higher one for enzyme–substrate complexes.

In view of this observation of two pK_{app} values, interpretation of the available data in terms of ion pair formation and related conformational changes may be formulated as a complex equilibrium, shown in figure 10. The mechanism for this equilibrium includes two ionization

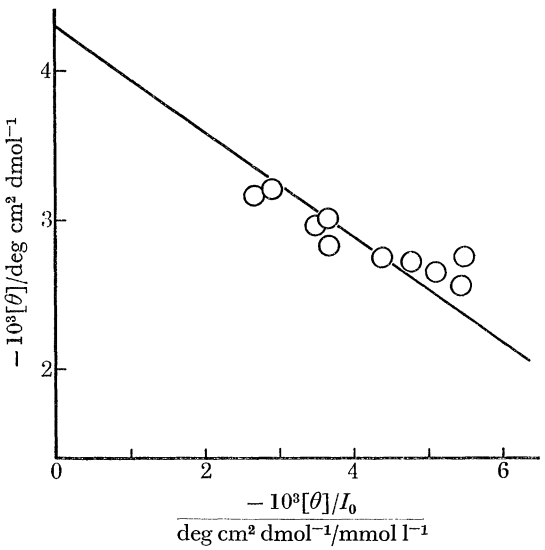

FIGURE 8

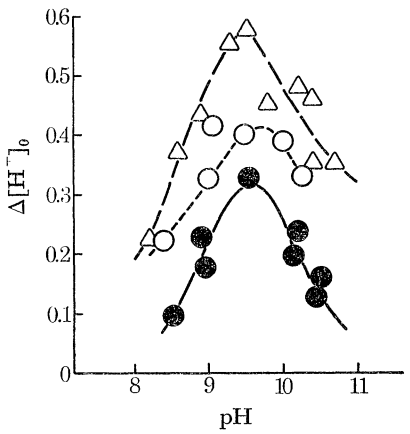

FIGURE 9

FIGURE 8. The effect of concentration of the specific inhibitor indole on the circular dichroism of α-chymotrypsin. Measurements of ellipticity (θ) were made at pH 9.0 and room temperature (see legend of figure 2 for experimental details). I_0 is the initial concentration of inhibitor.

FIGURE 9. The pH dependence of hydrogen ion uptake by chymotrypsin upon binding of substrate or inhibitor. Values are plotted as $\Delta[H^+]_0$, the observed proton uptake extrapolated to infinite substrate or inhibitor concentration. Δ, δ-chymotrypsin and indole; ○, α-chymotrypsin and *N*-acetyl-D-tryptophanamide; ●, δ-chymotrypsin and *N*-acetyl-L-tryptophanamide. Measurements were made at 25.0 ± 0.1 °C on a Radiometer pH meter with combination glass electrodes. The initial solution concentration of enzyme was 0.7 μmol l^{-1} and of substrate or inhibitor between 2 and 18 mmol l^{-1}.

constants for the α-amino group of Ile-16 as it exists in the two conformational forms: K_A, pertaining to the conformation in which the ion pair is intact, and K_I, pertaining to the conformation in which the ion pair is not formed. This latter form is the one that is considered to be characteristic of chymotrypsinogen and the high pH form of the enzyme. The mechanism also includes two conformational equilibrium constants, K_H and K_L, pertaining to the enzyme conformations at high and at low pH, respectively.

It is of interest to compare the information which we have obtained from chemical and physical studies of the enzyme in solution with the structural information obtained by Dr Blow and co-workers (Matthews, Sigler, Henderson & Blow 1967; Sigler, Blow, Matthews & Henderson 1968) with crystalline α-chymotrypsin at pH 4.2. An insight into the way in which ionization state of the isoleucine α-amino group, enzyme conformations, and the enzyme–substrate dissociation constant are related is suggested by the X-ray diffraction results. These results indicate that in the crystalline enzyme at pH 4.2 (the low pH conformation), the α-amino group of Ile-16 and the carboxyl group of Asp-194 point away from solvent and form an internal ion pair.

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Indeed, it is to be expected that at high pH, where the α-amino group does not carry a charge, this negatively charged carboxyl group would point into solvent,† since a very large unfavourable free energy is required to remove a negatively charged group from solvent in the absence of concomitant ion pair formation (Tanford & Kirkwood 1957). Inspection of the structure of chymotrypsin indicated to Blow and co-workers (Sigler *et al.* 1968) that if this negatively charged carboxyl group of Asp-194 did point into solvent, it would interfere with substrate binding in the active site of chymotrypsin.

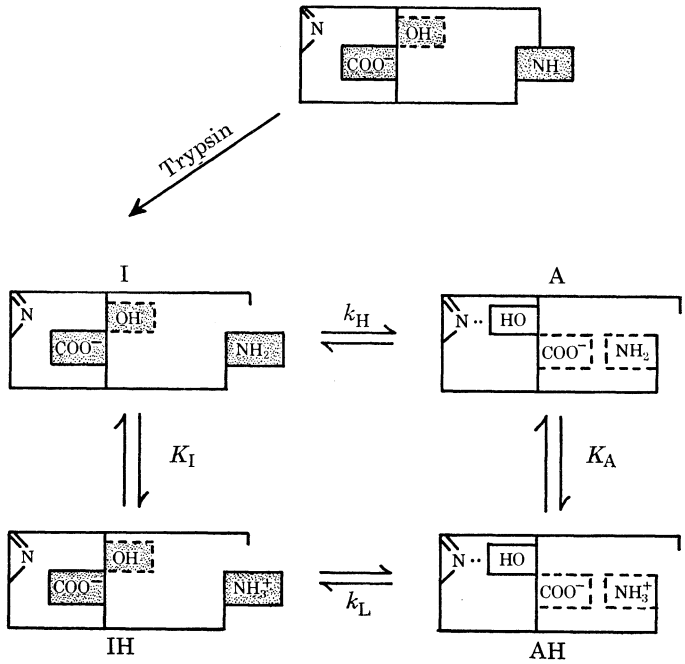

FIGURE 10. Mechanisms for the complex equilibria between chymotrypsin conformation forms, showing the ion pair formation that appears to be crucial for catalytic activity. Chymotrypsinogen (illustrated at top of diagram) is converted to chymotrypsin by action of trypsin. Two conformational forms of the enzyme are represented: I, the inactive form, and A, the active form. The conformational equilibrium constants K_H and K_L pertain to high and low (neutral) pH conditions, respectively; the ionization equilibrium constants K_I and K_A pertain to the α-amino group of Ile-16 in the two enzyme conformational forms. The diagram illustrates the idea that ion pair formation between the protonated α-amino group of Ile-16 and the negatively charged carboxyl group of Asp-194 is required in order to maintain the enzyme in the conformational form required for catalytic activity.

From the X-ray and solution studies of chymotrypsin, then, there emerges a detailed picture of chemical and structural changes associated with the processes of enzyme activation and enzyme catalysed reaction. It is known that in chymotrypsinogen and chymotrypsin at high pH, the α-amino group of Ile-16 is uncharged, and it is suggested that the carboxyl group of Asp-194 is pointing into the catalytic site,† thereby preventing substrate from binding. Structural considerations that make it unlikely for amino acid side chains to be in the *cis* configuration indicate that when the carboxyl group of Asp-194 points into solvent,† the adjacent serine residue is positioned so as to preclude the spatial relation between Ser-195 and His-57 that is considered necessary for catalytic activity. In agreement with this expectation is the fact that the

† A recent interpretation of the high resolution electron density map of chymotrypsinogen by Kraut, Wright, Xuong & Freer indicates that the movement of Asp-194 is more complex than described in this paper. We are grateful to Dr H. T. Wright for this information.

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catalytic centre of chymotrypsinogen is completely unreactive. When chymotrypsinogen is activated at neutral pH, or when the high pH form of chymotrypsin is brought to neutral pH, the isoleucine α-amino group acquires a positive charge, thereby allowing formation of an internal ion pair with the negatively charged carboxyl group of Asp-194. This process unblocks the substrate binding site and re-establishes the spatial relation between His-57 and Ser-195 that is considered necessary for catalytic activity. When the pH of the chymotrypsin solution is increased, this process reverses itself, thus accounting for the pH dependence of the enzymic reactions at alkaline pH.

(*b*) *The* pH *dependence of chymotrypsin catalysed reactions in the neutral* pH *region*

Reaction rates in the lower pH region appear to be controlled, as discussed above, by the ionization of imidazole nitrogen of His-57, an ionization characterized by a pK_{app} of about 7. In our work at neutral pH and below, intermediates in the reactions were detected by observation of the spectral changes of the enzyme near 290 nm which we found to accompany chymotrypsin catalysed reactions (Wootton & Hess 1962; Moon *et al.* 1965). We have also followed an approach, previously used by Bernhard & Gutfreund and associates (Bernhard & Gutfreund 1965; Bernhard, Lau & Noller 1965; Bernhard, Lee & Tashjian 1966), which makes

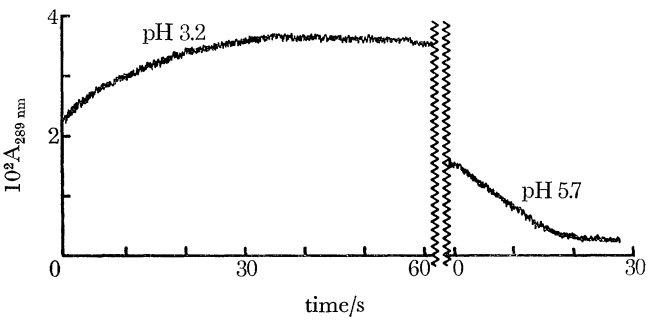

FIGURE 11. Spectrophotometric trace of a stopped flow measurement, showing individual steps in the chymotrypsin catalysed hydrolysis of *N*-acetyl-L-phenylalanine methyl ester. Absorbance (*A*) was measured as a function of time on a Cary spectrophotometer at 25 °C. At a specific time during the interval in which *A* is constant, the pH was changed from 3.2 to 5.7 in order to make the time dependent spectral changes more quickly measurable. Initial solution concentrations were 37 μmol l^{-1} of α-chymotrypsin and 17.2 mmol l^{-1} of substrate. Ionic strength was 0.4 mol l^{-1}.

use of chromophoric substrates or inhibitors whose spectra are perturbed upon binding to the enzyme. Reproduced in figure 11 is an oscilloscope trace obtained in a measurement, by a stopped flow technique, of absorbance changes near 290 nm during the chymotrypsin catalysed hydrolysis of a specific substrate ester, *N*-acetylphenylalanine methyl ester. Four distinct phases of the reaction are discernible in the experiment: (1) An initial rapid increase in absorbance, seen as a jump in the oscilloscope reading but not shown in this trace. This increase is completed in less than 3 ms and therefore is too fast to be measured by stopped flow techniques. We consider this step to be due to the reversible formation of enzyme–substrate complexes. Our investigations of this phase of chymotrypsin catalysed reactions by temperature jump experiments will be mentioned later. (2) A slower increase in absorbance, interpreted as evidence of the formation of a steady state intermediate. The rate of formation of this intermediate can be measured by stopped flow techniques. (3) A period of time corresponding to the steady state, during which the absorbance of the reaction mixture does not change. (4) A slow decrease in

absorbance, which may be identified with a decay of the steady state intermediate to give free enzyme and product. From progress curves such as this, three parameters can be evaluated when the conditions are chosen so that the initial substrate concentration is much greater than initial enzyme concentration. Under these conditions, the observed rate constants for formation of the steady state intermediate is a simple function of initial substrate concentration, S_0; the enzyme–substrate dissociation constant, K_S; and the rate constants for formation and for decay of the steady state intermediate. In terms of the mechanism shown in equation (1):

$$k_{obs} = \frac{k_{23}\, S_0}{S_0 + K_S} + k_{34}. \tag{4}$$

For a study of the pH dependence of the individual rate constants, we chose to work with the substrate acetyl-L-leucine methyl ester. The reason for choosing this substrate rather than the esters of aromatic amino acids is that the rate constants involved are somewhat smaller and can be measured with greater precision. The catalytic hydrolysis of acetyl-L-leucine methyl ester by chymotrypsin is still quite efficient, however, and the results are representative of what we have measured with less precision in the chymotrypsin catalysed hydrolysis of the esters of aromatic amino acids.

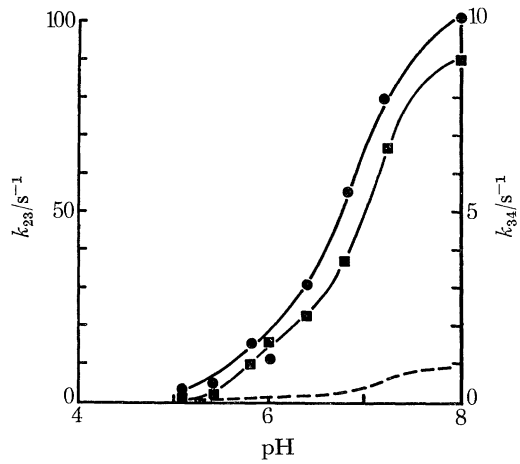

FIGURE 12. The pH dependence of rate constants pertaining to bond breaking steps in the chymotrypsin catalysed hydrolysis of acetyl-L-leucine methyl ester. ●, k_{23}; ■, k_{34} (see equation (1)). The dashed line shows k_{34} plotted on the scale drawn for k_{23}. Stopped flow measurements were performed according to a previously described (Brandt *et al.* 1967) proflavin displacement method in which change in concentration of an enzyme–proflavin complex is followed at 465 nm. Solutions buffered with 0.1 mol l^{-1} acetate or phosphate contained KCl to give an ionic strength of 0.4 mol l^{-1}. Temperature was 25 °C.

Values of k_{23} and k_{34} (and also K_S) were evaluated from plots of k_{obs} against k_{obs}/S_0 by means of a digital computer program based on the relation:

$$k_{obs} = \frac{k_{23}S_0}{S_0 + K_S[1 + (F_0/K_{EF})]} + k_{34},$$

where k_{obs} is the observed rate, F_0 is initial proflavin concentration (60 μmol l^{-1}), K_{EF} is the enzyme–proflavin dissociation constant, and S_0 is initial substrate concentration (2.5 to 80 mmol l^{-1}).

The acetyl-L-leucine methyl ester data shown in figure 12 indicate that both of the measured rate constants are controlled by an ionizing group of the enzyme with $pK_{app} \sim 7$. As defined by the mechanism in equation (1), these rate constants are k_{23} (solid circles), pertaining to the formation of the steady state intermediate EP_2, and k_{34} (solid squares), pertaining to the decomposition of EP_2 to free enzyme and product. The dashed line on the bottom of the graph

represents the pH dependence of k_{34} drawn on the same scale used for k_{23}. It is apparent that k_{34} is much smaller than k_{23}; therefore, it characterizes the rate limiting step of the reaction, and is the only rate constant that is measured in steady state kinetic experiments. It may be noted that both k_{23} and k_{34} are presumed to pertain to bond breaking steps involved in the reaction: the liberation of alcohol, which is P_1, the first product in the mechanism of equation (1); and the liberation of acid, which is P_2, the second product.

TABLE 1. RATE AND EQUILIBRIUM CONSTANTS PERTAINING TO α-CHYMOTRYPSIN CATALYSED HYDROLYSES OF SPECIFIC SUBSTRATE ESTERS AT pH 5.0 AND 25 °C.

Stopped flow measurements, yielding k_{23} and K_S, were made by a proflavin displacement method (Brandt, Himoe & Hess 1967), and steady kinetic measurements, yielding k_{cat} and $K_{m,app}$ were determined by pH-stat titration under conditions of excess initial substrate concentration.

compound	k_{23}/s^{-1}	k_{34}/s^{-1}	K'_S†/mmol l^{-1}	$K_{m,app}$/mmol l^{-1}	b‡
acetyl-L-Trp ethyl ester	35 ± 9	0.84	2.1 ± 0.6	0.08	1.3
acetyl-L-Phe ethyl ester	13 ± 2	2.2	7.3 ± 1.5	1.3	1.2
acetyl-L-Tyr ethyl ester	83 ± 24	3.1	18 ± 6	0.8	0.8
acetyl-L-Leu methyl ester	3.2 ± 0.4	0.19	93 ± 11	—	—

† Calculated from observed slope $= K'_S(1+F_0/K_{EF})$ where $F_0 = 50$ μmol l^{-1}; $K_{EF} = 40$ μmol l^{-1}.
‡ $b = [k_{23}/K'_S]\,[k_{cat}/K_{m,app}]^{-1}$.

A comparison of rate and equilibrium constants for the hydrolysis of acetyl-L-leucine methyl ester at pH 5 and 25 °C, with corresponding parameters for the hydrolyses of three aromatic ester substrates is shown in table 1. It is apparent that in all cases, k_{34} is much smaller than k_{23}, which suggests the generalization that in ester hydrolysis the rate limiting step is the breakdown of the steady state intermediate. This suggestion was made previously by Hartley & Kilby (1954) and Gutfreund & Sturtevant (1956) on the basis of studies with a model substrate, *p*-nitrophenyl acetate, and by Bender & Kézdy (1965) on the basis of steady state kinetic measurements with specific substrates.

3. INVESTIGATIONS IN PROGRESS

Results of some recent investigations, still in progress, will indicate the directions being taken in our experiments.

Figure 13 shows results of our investigation of the chymotrypsin catalysed hydrolysis of furylacryloyltryptophanamide, using relaxation, flow, and steady state kinetic experiments. The material was a gift of Dr Bernhard, who introduced the use of the furylacryloyl group (Bernhard *et al.* 1965) in order to be able to detect the formation of enzyme–substrate complexes, for the spectrum of this chromophore is perturbed upon binding to chymotrypsin. The experiments shown in this figure were performed at pH 7.4 and 15 °C. It is evident that three different processes are being measured. The first process, measured by the temperature jump technique, occurs in the microsecond region of the time scale. The second process, measured by stopped flow methods, takes about a hundred times longer. The third process, measured by the steady state liberation of ammonia, takes about a thousand times longer than the second process.

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A mechanism consistent with these data is shown in figure 14. The magnitudes of the observed rate constants and their concentration dependencies help identify the observed processes with particular steps in the reaction. The first step, measured by temperature jump, appears to be the formation of a first enzyme–substrate complex, ES_1. This process is essentially pH independent. The second process, which we have measured by flow techniques, leads to the formation of ES_2 and is pH dependent. We do not yet have enough data to be certain about the pK_{app} which controls this step. Product is not released concomitant with this step. This appears to be

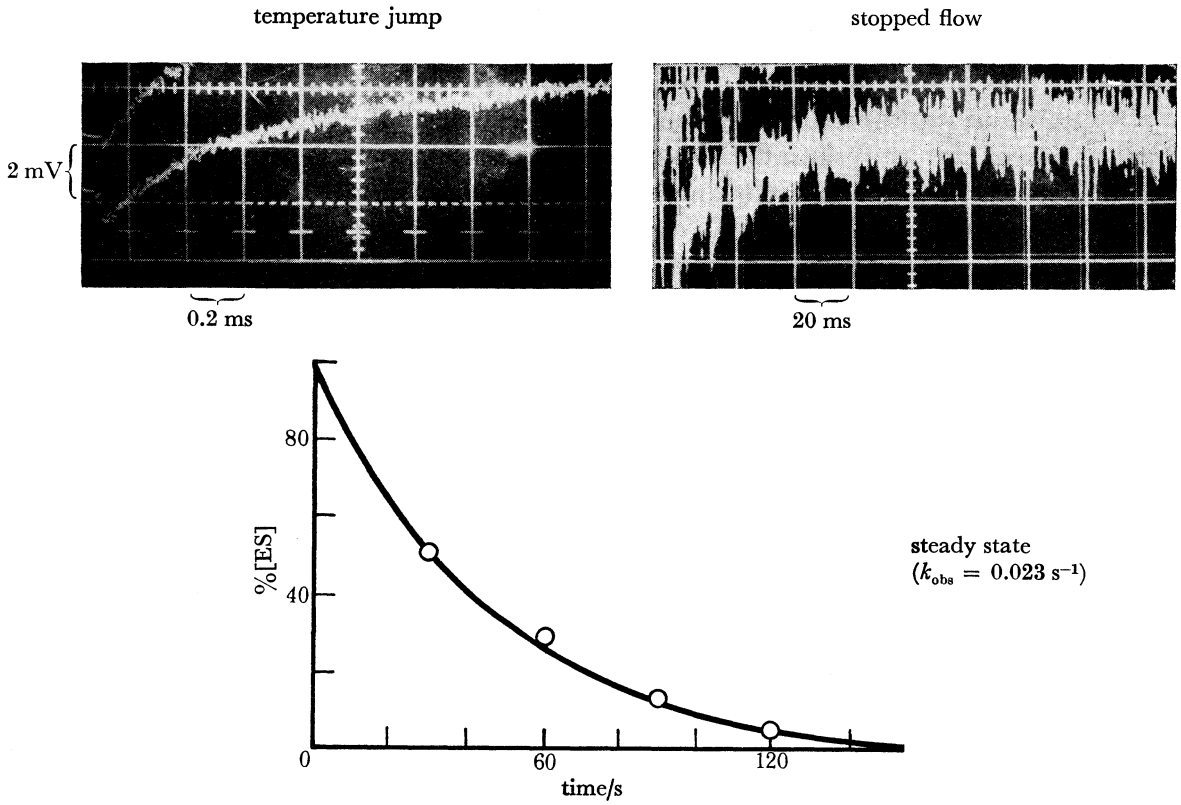

FIGURE 13. Kinetics of the chymotrypsin catalysed hydrolysis of furylacryloyl-L-tryptophanamide at pH 7.4 and 15 °C, as measured by temperature jump, stopped flow, and steady state techniques. Typical results obtained by these three methods are illustrated. (See discussion in text.)

a significant difference in the mechanisms for chymotrypsin catalysed hydrolysis of esters and amides. It may be recalled that in the catalytic hydrolysis of esters, an ionizing group with $pK_{app} \sim 7$ controls both bond breaking steps; in amide hydrolysis, there appears to be a step controlled by an ionizing group, which is not associated with product release. This step is followed by the slowest step in the reaction, which is associated with ammonia release, and which is controlled by an ionizing group with $pK_{app} \sim 7$. It is this last step, and only this step, that is measured in steady state kinetic experiments.

The diagram shown in figure 14 has been simplified by omission of conformation and ionization equilibria for all species except the free enzyme. The conformational equilibria may be ignored in these experiments, since the enzyme is expected to be mainly in the active or low pH conformation at pH 7.4 and 15 °C. However, under physiological conditions, pH 8 and 37 °C, the equilibria between enzyme conformations become important and must be considered.

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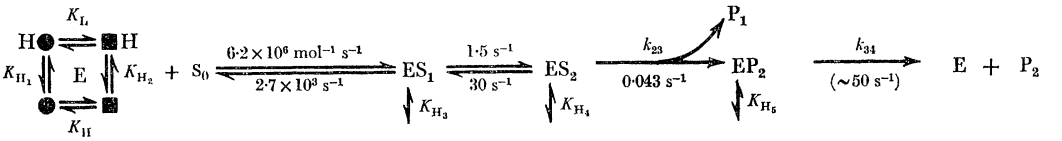

FIGURE 14. A mechanism for chymotrypsin catalysed hydrolysis of substrate amides that is consistent with the results of relaxation, stopped flow, and steady state kinetic experiments with furylacryloyl-tryptophanamide, such as are shown in figure 13. Conformation and ionization equilibria are diagrammed only for the free enzyme, but are presumed to exist for the other species as well. Ionization constants are indicated as K_{H_1}, K_{H_2}, etc., and conformation constants K_L and K_H refer to equilibria at low and high pH levels, respectively. S_0 is initial substrate concentration, present in excess of enzyme, E, and taken as constant; ES_1 and ES_2 are complexes, EP_2 is an enzyme–substrate compound; and products P_1 and P_2 are, in this reaction, ammonia and furylacryloyl-tryptophan, respectively.

One technique we are exploring with the object of obtaining information about the substrate binding sites of chymotrypsin is the measurement of fluorescence of the enzyme as a function of pH. Figure 15 shows absolute quantum yield plotted against pH for chymotrypsin (solid line). The excitation and emission wavelengths (300 and 340 nm respectively) are characteristic of the fluorescence of tryptophan. It is apparent that the fluorescence of chymotrypsin decreases with decreasing pH as an ionizing group of the enzyme with pK_{app} ~ 6 gains a proton. These measurements are similar to data obtained previously by Katchalski and co-workers (Shinitzky,

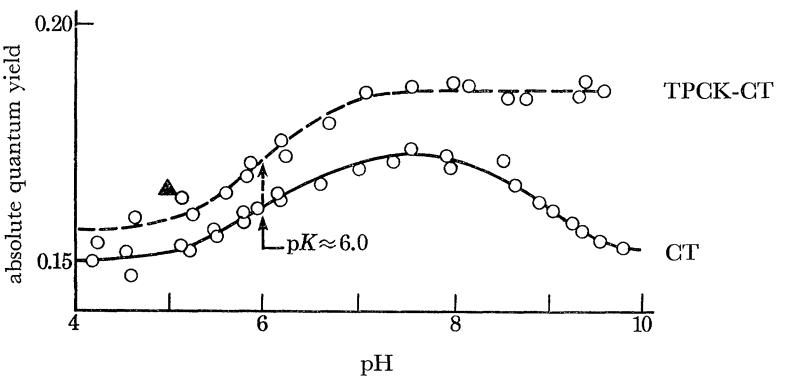

FIGURE 15. The quenching of the fluorescence of chymotrypsin as a function of pH and substrate binding. Values of absolute quantum yield, measured at 340 nm and 25 °C, are shown for δ-chymotrypsin (solid line), and for the derivative TPCK-CT, where TPCK is L-1-tosylamido-2-phenylethyl chloromethyl ketone and CT is δ-chymotrypsin (dashed line). The triangle represents a measurement of the complex between δ-chymotrypsin and the inhibitor *N*-acetyl-D-phenylalaninamide (see text). Measurements were made on an Aminco-Bowman Spectrophotofluorimeter, using slit program no. 3. Experimental solutions were buffered with acetate, phosphate, or pyrophosphate, and had ionic strengths of 0.4 mol l^{-1}.

Katchalski, Grisaro & Sharon 1966; Shinitzky & Goldman 1967). According to model studies of these investigators, this fluorescence quenching of tryptophan occurs when a nearby histidine residue becomes protonated and a complex is formed by interaction of the protonated imidazole ring of the histidine with an indole ring of tryptophan. This complex is broken when the imidazole ionizes, and this leads to increase in fluorescence. There are two histidine residues in chymotrypsin, and we wanted to find out whether His-57 in the active site of the enzyme is the one responsible for the fluorescence quenching. A way in which we might find this out was suggested by the work of Ong, Shaw & Schoellmann (1965) which showed that His-57 reacts specifically with an α-amino ketone derivative of a substrate, *N*-tosyl-L-phenylalanine. It is known that the reaction of this reagent with His-57 lowers the pK of this residue by 0.8 pH unit (Glick 1968). The dashed line in the figure gives the pH dependence of absolute quantum

yield obtained with this modified chymotrypsin molecule. It is apparent from the data in the figure that the pH dependence of this chymotrypsin derivative is exactly the same, in the pertinent pH region, as that of chymotrypsin, indicating that His-57 is not involved in the pH dependent change in fluorescence of the enzyme. Presumably, therefore, the observed fluorescence quenching is an effect of the ionization of the other histidine, which is residue 40. The solid triangle shown in the figure gives the absolute quantum yield for chymotrypsin in complex with the inhibitor *N*-acetyl-D-phenylalaninamide at pH 5. This measurement was obtained as part of an experiment in which binding constants pertaining to this inhibitor and chymotrypsin were determined over a range of pH from measurements of fluorescence as a function of inhibitor concentration. Similar data were obtained with the specific substrate *N*-acetyl-L-phenylalaninamide. These data indicate that in the enzyme–inhibitor or enzyme–substrate complex, the pH dependent fluorescence changes of the enzyme have not been modified. One explanation of these results is that the binding of inhibitor or substrate does not interfere with the formation of the complex between histidine and an indole ring of tryptophan.

This paper has presented a brief survey of some of the approaches used to investigate the activity of chymotrypsin, and of some of the results that have been obtained. If there is anything definite to be said about the activity of this enzyme, it is that at this time very little is known about the efficiency and specificity so characteristic of reactions catalysed by chymotrypsin, and of all other biological reactions. These properties of enzymes apparently depend on the interaction of the steps associated with binding of substrate and the transformation of substrates to products. A great many parameters are involved, and it will take a variety of techniques and approaches to sort them out.

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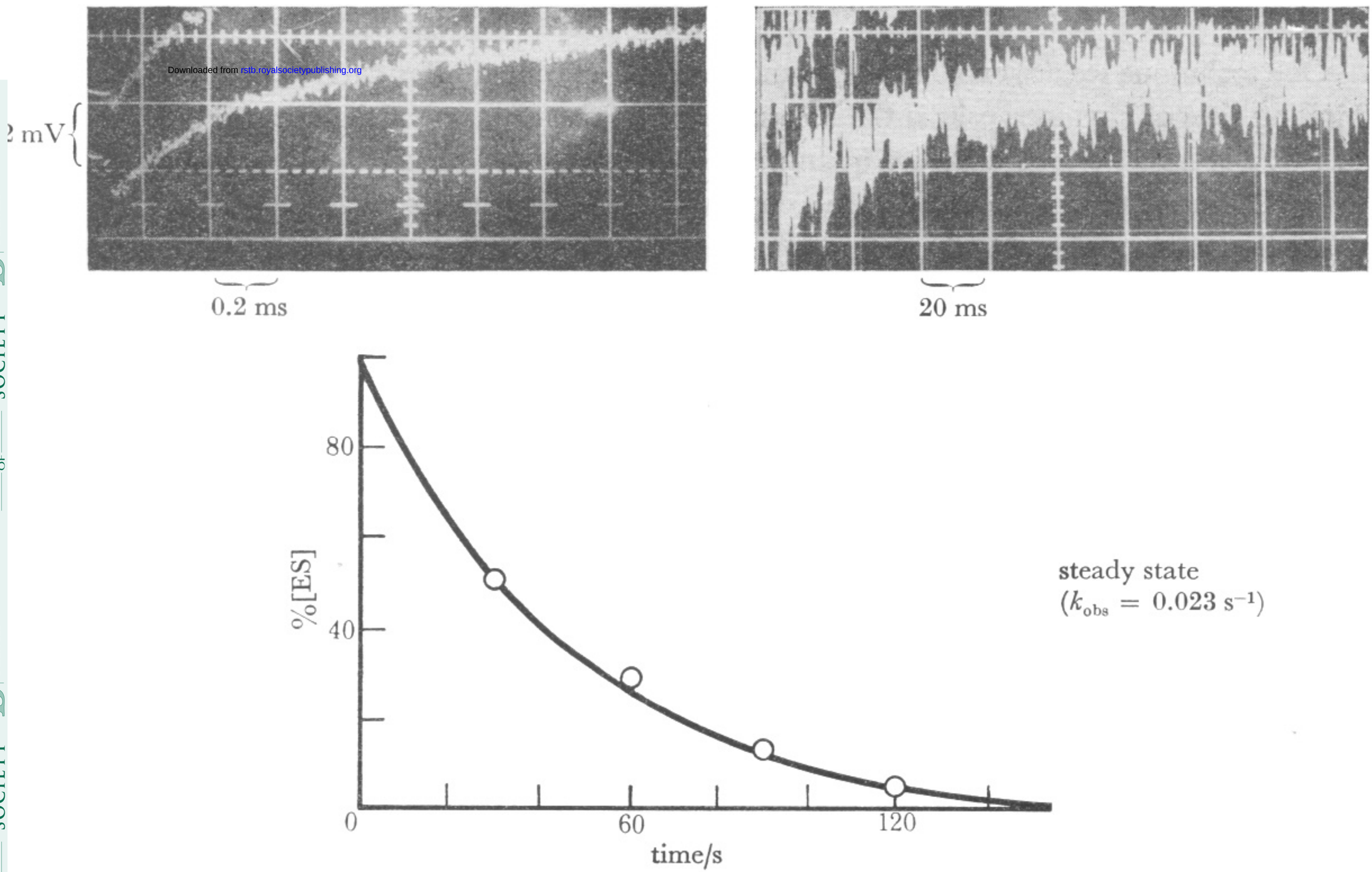

FIGURE 13. Kinetics of the chymotrypsin catalysed hydrolysis of furylacryloyl-L-tryptophanamide at pH 7.4 and 15 °C, as measured by temperature jump, stopped flow, and steady state techniques. Typical results obtained by these three methods are illustrated. (See discussion in text.)