Concerning the Mechanism of Autolysis of α-Chymotrypsin*

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ABSTRACT: The mechanism of autolysis of α-chymotrypsin has been investigated at pH values from 3 to 9.7 in terms of changes in the kinetic parameters. The results illustrate the following points: (a) commercial α-chymotrypsin does not appear to be a mixture of two kinetically distinguishable species; (b) the mechanism of autolysis can, at about 30° and at a protein concentration of about 1.0 mg/ml, be explained by an apparent second-order inactivation process, although at concentrations higher than 5 mg/ml it is complex; (c) at 1.0 mg/ml and about 40° the inactivation process is apparently mixed and follows first- and second-order kinetics; and (d) the pH *vs.* apparent autolytic rate profile gives a sharp bell-shaped curve with a maximum near pH 9.1. The results can be explained on the basis of a conformational equilibrium between two enzyme species, accompanied by digestion of one form by the other. The process is akin to zymogen activation and is consistent with a "one-by-one" mechanism.

In spite of the proliferation of studies dealing with the mechanism of chymotrypsin-catalyzed digestion of proteins, little work has been done on the important question of kinetics and mechanism of enzymatic selfdigestion, or autolysis. Earlier studies have shown that autolysis is accompanied by the degradation of a protein into less complex molecules (Wells and Benson, 1907; Haehn and Leopold, 1934; Herriott and Northrop, 1934; Herriott *et al.*, 1940; Wu and Laskowski, 1956); and some workers have reported active fragments as a result of autolysis or after digestion of one enzyme by another (Northrop *et al.*, 1948; Cunningham and Neurath, 1953; Perlman, 1954; Wainfan and Hess, 1960; Ginsberg and Schachman, 1960). Recent interpretations of the mechanism of autolysis of α-chymotrypsin are based either on short incubation periods of autolysis (Hofstee, 1965), or use high enzyme concentrations (Bender *et al.*, 1964), where the enzyme is known to polymerize (Rao and Kegeles, 1958; Martin and Niemann, 1958).

In the work reported here, an attempt has been made to elucidate the kinetics and mechanism of autodigestion of the enzyme α-chymotrypsin. An important factor in our studies is the use of a new and rapid kinetic assay (Kumar and Hein, 1969), which measures the continuous progress of autolysis in terms of changes in kinetic constants. Therefore, the difficulties in obtaining kinetic constants from conventional activity assays (using the initial rate, where $[S] \gg K_{0(app)}$) or all-or-none assays (Erlanger *et al.*, 1964) are circumvented.

The first quantitative studies on the autolysis of chymotrypsin were those of Chernikov in 1955. Subsequent work has consistently indicated that autolysis proceeds as a first-order reaction (Hofstee, 1965; Bender *et al.*, 1964; Erlanger *et al.*, 1964). Our kinetic data, which are consistent with a second-order process, lead us to suggest a mechanism for autolysis which involves the attack of one molecule on another as the rate-limiting step.

Experimental Procedures

Materials. Three-times-crystallized salt-free α-chymotrypsin was purchased from Worthington (lots CDI 6125-6 and CDI 6051-2) and from Mann (lot M-2). *N*-Acetyl-L-tyrosine ethyl ester was also obtained from Mann. The enzyme and substrate were used without further purification. Ninhydrin was from Aldrich. The Tris and 2-amino-2-methylpropane-1,3-diol obtained from Fisher were twice crystallized from absolute ethanol. The 2-hydroxyethylamine (bp 172–173°) and 2-methoxyethanol (methyl Cellosolve, bp 124–125°) were freshly distilled for use. All of the other chemicals were of reagent grade quality.

Apparatus. The kinetic assays were performed on a Beckman Model DK-1 double-beam recording spectrophotometer with a deuterium lamp and a thermostated cell compartment. A Bausch and Lomb Model 28 spectrophotometer was employed for the measurement, at 570 mμ, of the optical densities of the ninhydrin-positive species during autolysis. For determining the progress of autolysis as a function of base uptake, a pH-Stat (International Instrument Co., Calif.) was used.

A Leeds and Northrop pH meter (type 7041) with semimicro electrodes was used to measure the pH of the buffer solutions, employing Beckman pH 4.0, 7.0, and 10.0 buffers as standards.

Methods. SOLUTIONS. Stock solutions of α-chymotrypsin (10 mg/ml) were prepared in 10^{-3} N HCl–0.1 M NaCl and kept in ice. Substrate solutions were made directly in 0.1 M (pH 7.0) phosphate buffer, and were approximately 2 mM. The exact concentrations of the enzyme stock and substrate solutions were calculated as described previously (Kumar and Hein, 1969).

Kinetic Assay Method. The method for the determination of the difference extinction coefficient between *N*-acetyl-L-tyrosine ethyl ester and *N*-acetyl-L-tyrosine at 237 mμ, and

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the procedure for the kinetic assay have been described previously (Kumar and Hein, 1969).

Buffer Compositions for the Autolysis Experiments. Univalent buffers (about 0.1 M) were used for the incubation of the enzyme at each of the pH values. The ionic strength was generally maintained at about 0.1 by the addition of 1.0 M NaCl. The buffers employed at each pH value were: 0.001 M HCl–0.1 M NaCl (pH 3.1), 0.1 M sodium acetate–acetic acid (pH 5.05), 0.1 M Tris-HCl (pH 7.0–8.8), 0.1 M 2-amino-2-methylpropane-1,3-diol-HCl (pH 8.0–9.15), 0.1 M glycine–NaOH (pH 9.25–9.7), and 0.1 M 2-hydroxyethylamine-HCl (pH 9.55).

Incubation at pH Value. For each incubation, 9.0 ml of 0.11 M buffer of the appropriate pH was placed in a 25-ml flask and equilibrated at the required temperature for at least 5 min. Also equilibrated at the same temperature was the enzyme stock. Then 1.0 ml of the enzyme solution was transferred to the buffer solution — the time of mixing being taken as the mean of the time taken for the complete transfer of the enzyme solution. The flask was gently shaken, stoppered, and incubated in the constant-temperature bath. (The protein concentration in the incubation mixture was approximately 1.0 mg/ml.) Volumes of 20–100 μl (depending upon the extent of autolysis) of the incubation mixture were removed for kinetic assay at various time intervals. The same volume of buffer was generally added to the reference cell. The actual volume added to the cells for the assay was calculated from the weight of the same amount of water, with a density correction for 30°. The concentration of the active enzyme in the incubation mixture ($[E]_{inc}$) was obtained by multiplying the kinetic assay value by the dilution factor. The kinetic assay value ($[E]_{assay}$) was obtained by dividing V_{max} by k_0 (130 sec^{-1}) at pH 7, 30°, and 0.1 M phosphate.

The pH of the incubation mixture was recorded at the end of the incubation period and differed from the original value by no more than 0.05 pH unit.

Base-Uptake Measurement. α-Chymotrypsin (about 0.75 μmole) was dissolved in 10 or 20 ml of 0.1 M NaCl (adjusted to about pH 5.0) and placed in the thermostated reaction vessel of the pH-Stat. The solution was allowed to equilibrate at 30 or 40° for 5 min, and then, by the addition of 1.0 N NaOH, the pH was quickly (in less than 30 sec) adjusted to the desired value. The subsequent continuous base (0.1 N NaOH) addition, to maintain the proper pH, was recorded automatically.

In the measurements of the blanks, without the enzyme, the same procedure was employed. Carbon dioxide free distilled water was used for all solutions; and a wet stream of nitrogen was maintained over the solution in the reaction vessel.

Measurement of Ninhydrin-Positive Species. The appearance of ninhydrin-positive materials during the autolysis was followed quantitatively according to the procedure of Rosen (1957). Enzyme stock (1.0 ml; concentration 10.0 mg/ml) was added to 9.0 ml of 0.11 M 2-amino-2-methylpropane-1,3-diol (pH 9.15), after both were equilibrated at 30°. At certain time intervals, 100 μl of the enzyme was withdrawn and added to 1 ml of acetate–cyanide solution (2.95 M acetate–0.23 mM cyanide, pH 5.4) in a 15-ml centrifuge tube. Then 0.5 ml of ninhydrin solution (3% w/v in 2-methoxyethanol) was added to the tube. The tube was firmly capped, shaken for 10–15 sec, and placed in a boiling-water bath for 15 min to complete the reaction. The tube was then removed from the bath, cooled in ice water, and 6.0 ml of a diluent solution (isopropyl alcohol–water, 1:1, v/v) was added. The precipitated and suspended protein was centrifuged. The centrifugate was poured into a volumetric flask and made up to 10 ml with the diluent solution. Then the optical density at 570 mμ was measured. DL-Phenylalanine, at concentrations comparable with the enzyme, was used as a standard, and an extinction coefficient of $1.4 \times 10^4\ M^{-1}\ cm^{-1}$ was obtained. The number of ninhydrin-positive groups was calculated on the basis of this extinction coefficient.

Interpretation of Data in Terms of Modified Rate Constants. In the conventional Michaelis–Menten scheme, the rate at which a substrate is hydrolyzed is represented as

$$\text{rate} = -\frac{d[S]}{dt} = \frac{k_0[E][S]}{K_{0(app)} + [S]}$$

and

$$\frac{d(\text{rate})}{dt} = f(k_0[E], K_{0(app)})$$

Since the activity change during enzyme modification is independent of substrate concentration ($[S] \gg K_{0(app)}$), it can be related to alterations in $k_0[E]$ and $K_{0(app)}$. If active, native enzyme and inactive enzyme are present in the autolysis mixture, then the observed change is a decrease in $k_0[E]$, and the change in activity can be attributed to a decrease in [E]. If a modified enzyme species is produced which had kinetic properties different from the native enzyme, then one would expect that both $K_{0(app)}$ and $k_0[E]$ would appear altered in the assay.

Since all our results show that $K_{0(app)}$ remains constant during inactivation, we can conclude that either no active modified forms are formed or that any modified form has a $K_{0(app)}$ value identical with the native enzyme. In the absence of any evidence for a modified active enzyme species from either our studies or previous ones, we have interpreted the changes in $k_0[E]$ to reflect changes in enzyme concentration. This interpretation is further supported since the assumption permits the data to be analyzed in terms of a simple kinetic scheme. (The term "inactive" here refers to the enzyme with weak catalytic properties; too weak to be observed under the conditions of our kinetic assay.)

Results

Autolysis at 30°. The data in Figure 1A for pH 9.05 are typical of that obtained at all pH values when autolysis of α-chymotrypsin is measured in terms of changes in kinetic parameters. $[E]_{inc}$ has been calculated from $[E]_{assay}$ assuming k_0 to be constant (at a value of 130 sec^{-1}). It is noted that during the incubation time a continuing decrease in the concentration of active enzyme species occurs, while $K_{0(app)}$ remains constant.

Figure 1B shows a semilogarithmic plot of $[E]_{inc}$ against the time of incubation. The plot has a distinct curvature even where more than 70% of the enzyme has been autolyzed. Except at low pH values (5–7.9) the autolysis was followed until about 70% of the enzyme was no longer active. Evaluation of data when autolysis is greater than about 70% is

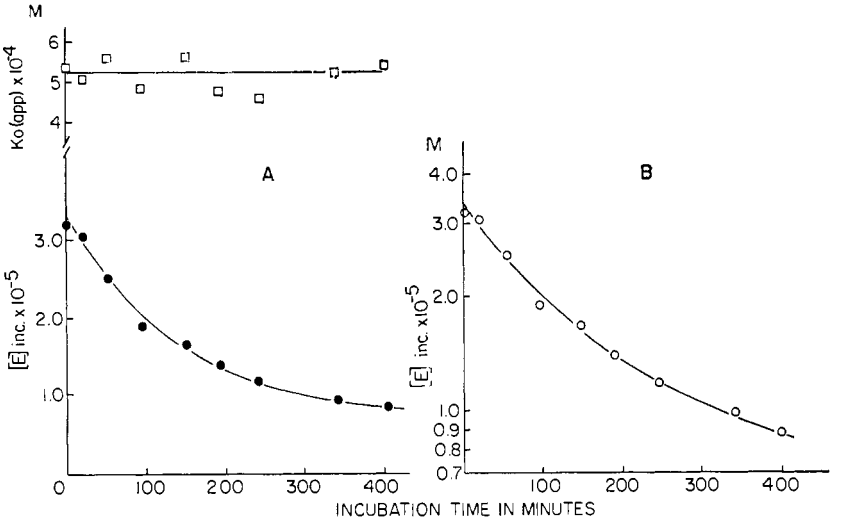

FIGURE 1: Autodigestion of α-chymotrypsin in 0.1 M 2-amino-2-methylpropane-1,3-diol-HCl (pH 9.05). Initial enzyme concentration in incubation mixture was ~1.0 mg/ml. $[E]_{inc}$ was calculated from $[E]_{assay}$. Concentrations of enzyme and *N*-acetyl-L-tyrosine ethyl ester in assay mixture were 0.212 μM and 2.1 mM, respectively. In A, (□) and (●) represent $K_{0(app)}$ and active enzyme concentration, at different periods of autolysis. Part B is a semilogarithmic plot of $[E]_{inc}$ from the data of A.

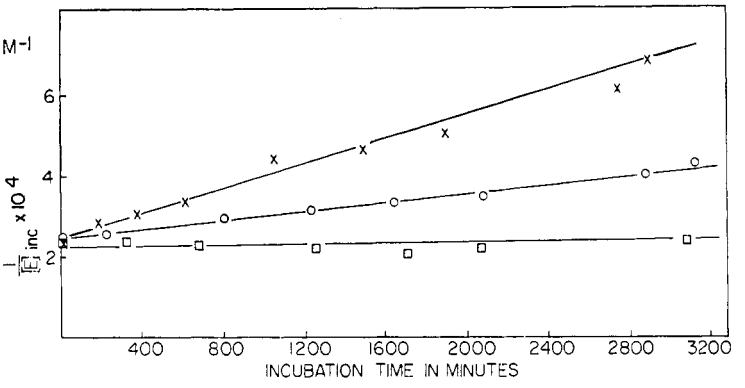

FIGURE 2: Second-order rate plots of $1/[E]_{inc}$ *vs.* time of incubation of α-chymotrypsin at pH 5.0 (□), 7.0 (○), and 7.9 (×), at 30° and ionic strength of ~0.1. Concentration of incubated enzyme at each pH was ~0.1 mg/ml. Buffers (0.1 M) were sodium acetate-acetic acid (pH 5.0) and Tris-HCl (pH 7.0 and 7.9). The values of apparent second-order rate constants (in M^{-1} min^{-1}) are: ~0 (pH 5.0), 5.0 (pH 7.0), and 16 (pH 7.9).

complicated by the presence of peptide fragments in solution which protect the enzyme against further inactivation (Chernikov, 1955).

Since the semilogarithmic plots at all pH values do not fit a straight line, it is apparent that the autolysis at these concentrations at 30° is not a unimolecular process. It was also impossible to fit the curve to two simultaneous first-order processes. Thus, it is unlikely that at these experimental conditions two forms of the enzyme are being inactivated at two distinct first-order rates.

Autolysis studies were carried out at eleven pH values between pH 5.0 and 9.7. Plots of $1/[E]_{inc}$ *vs.* time at some of these values are shown in Figures 2 and 3. The following observations can be made from these figures: (a) within the experimental accuracy, apparent second-order kinetics for autolysis are observed at each pH value. Any deviations from linearity become evident only after about 70% of the enzyme has been autolyzed; (b) the apparent second-order rate constants (slopes of the lines) increase from pH 5.0 to 9.05, and then decrease again up to 9.7; and (c) during the first 60 hr, autolysis is almost negligible at pH 5.0, and is less than 25% at pH 7.0. Therefore, in terms of kinetic properties, solutions of α-chymotrypsin are quite stable at room temperature for several hours.

Changes in the Apparent Second-Order Rate Constant $k_{(app)}$ with pH. The apparent second-order rate constants (as obtained from the slopes of the lines for the second-order inactivation) are plotted as a function of pH in Figure 4. It can be seen that there is very little autolysis at pH 5.0. The rate increases at higher pH values, reaches a maximum at about pH 9.0, and then decreases again. The enzyme appears to be more stable at pH 9.5 than at pH 8.8.

Supporting Evidence for Autolysis. The results showing the extent of autolysis presented in Figures 1–4 were substantiated by two other methods.

In one method, the progress of autolysis was followed by observing the change in concentration of a ninhydrin-reacting species at 570 mμ as a function of time. The results of such an experiment at pH 9.15 and at 30° are shown in Figure 5. The Δ optical density values shown in the figure have been obtained by subtracting the blank at zero time for the same concentration of enzyme. It is clear from the figure that the increase in optical density measured in the ninhydrin assay is paralleled by a decrease in the concentration of active enzyme measured in the kinetic assay. This is emphasized in the inset of Figure 5, where the percentage of inactivation of the enzyme is plotted against the optical density at 570 mμ, to yield a straight line.

The second method measured the base uptake, at constant pH, during autolysis. It was found that at pH 8.5, the increase in total base uptake was parallel to the decrease in enzyme concentration. Again, the percentage of loss of enzyme

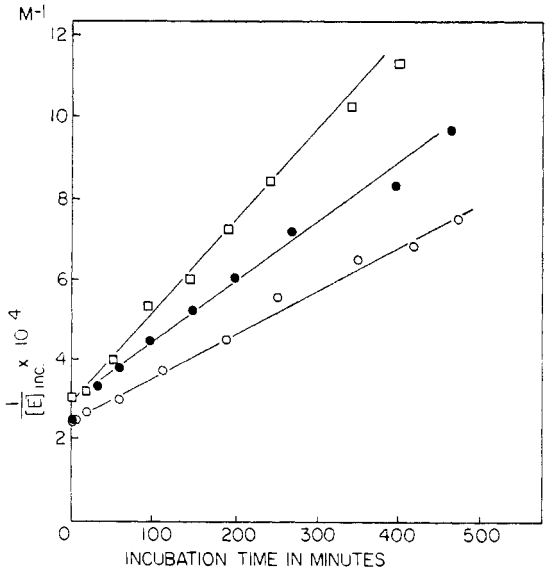

FIGURE 3: Plots of $1/[E]_{inc}$ *vs.* time of incubation of α-chymotrypsin at pH 8.8 (●), 9.05 (□), and 9.55 (○), at 30°, and ionic strength of ~0.1. Enzyme concentration at each pH was ~1.0 mg/ml. Buffers (0.1 M) were 2-amino-2-methylpropane-1,3-diol-HCl (pH 8.8 and 9.05) and 2-hydroxyethylamine-HCl (pH 9.55). The values of apparent second-order rate constants (in M^{-1} min^{-1}), are 152 (pH 8.8), 230 (pH 9.05), and 108 (pH 9.55).

activity was directly proportional to the increase in base uptake.

The results obtained by these two methods were utilized to calculate the number of peptide bonds broken during autolysis. In the absence of autolysis, 16–17 ninhydrin-positive groups were found per mole of enzyme, based upon an extinction coefficient of 1.4×10^4 M^{-1} cm^{-1} for D,L-phenylalanine at 570 mμ. At different time periods during autolysis, the number of new ninhydrin-positive species was calculated from the decrease in concentration of the enzyme

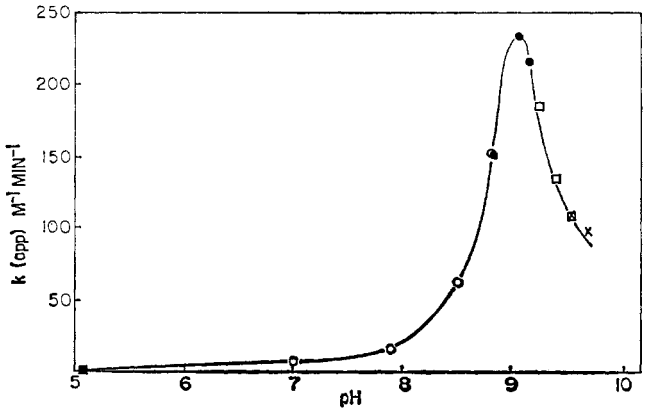

FIGURE 4: The variation of the apparent second-order rate constant for α-chymotrypsin autodigestion with pH. Enzyme concentrations were ~1.0 mg/ml, buffers were 0.1 M, ionic strength 0.1, and temperature 30°. Buffers were: sodium acetate–acetic acid (■), Tris-HCl (○), 2-amino-2-methylpropane-1,3-diol·HCl (●), glycine–NaOH (□) and 2-hydroxyethyl amine·HCl (×).Buffer overlaps are indicated by (◑) and (⊠).

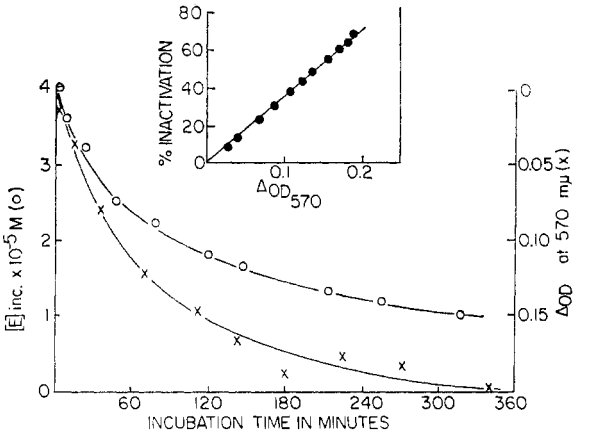

FIGURE 5: Autolysis of α-chymotrypsin at pH 9.15, 30°. Enzyme concentration was ~1.0 mg/ml. (○) and (×) represent, respectively, concentrations of active enzyme and ninhydrin-positive species (measured at 570 mμ). Figure 5 inset shows a plot of the per cent of enzyme inactivation *vs.* the optical density at 570 mμ, from the data taken from Figure 5. Details are given in Methods.

as measured by kinetic assay and increase in optical density at 570 mμ. These calculations showed approximately 40 new ninhydrin-reacting groups per mole of the enzyme. This suggests that perhaps hexapeptides (on the average) are produced during autolysis at pH 9.15. The base-uptake studies also led to similar conclusions.

Inactivation of α-Chymotrypsin at 40°. Figure 6 illustrates the inactivation process at pH 7.9 and 40° at two initial enzyme concentrations (0.1 and 1.0 mg per ml). The inactivation process at low protein concentrations appears as essentially first order (with an apparent rate constant of 1.40×10^{-3} min^{-1}), but at the higher protein concentration a pronounced deviation from the first-order behavior is observed. When about 90% of this protein has been autolyzed, the inactivation again appears to follow first-order kinetics. The latter part of the curve for the inactivation at the higher concentration has a slope very similar to that of the curve for the lower concentration. The inactivation process for the higher protein concentration cannot be described by two simultaneous first-order reactions. This contrasts with the results of Erlanger *et al.* (1964).

The data for the protein concentration of 1.0 mg/ml found in Figure 6 were plotted as a second-order function ($1/[E]_{inc}$ *vs. t*) in the inset of Figure 6. A second-order rate constant (9.0×10^2 M^{-1} min^{-1}) could be calculated from the initial linear portion of the graph. Using eq 6 (see Discussion) and the values of $k_1K_1K_2/[H^+]$ and $k_2K_1K_2/K[H^+]$ from the first- and second-order plots, respectively, a curve was constructed by the IBM 1620 computer for the inactivation of α-chymotrypsin at pH 7.9 and 40°. The results are shown in Figure 7.

The calculated and experimental values show excellent agreement, except during the latter stages of autolysis. The experimental values of $[E]_{inc}$ are slightly higher than those which were calculated. It is possible that at longer time periods accumulation of appreciable amounts of autolysis products protect the enzyme against inactivation.

The increase in base uptake at pH 7.9, 40° (at protein concentration of 1.0 mg/ml) was also compared with the decrease in the concentration of active enzyme under iden-

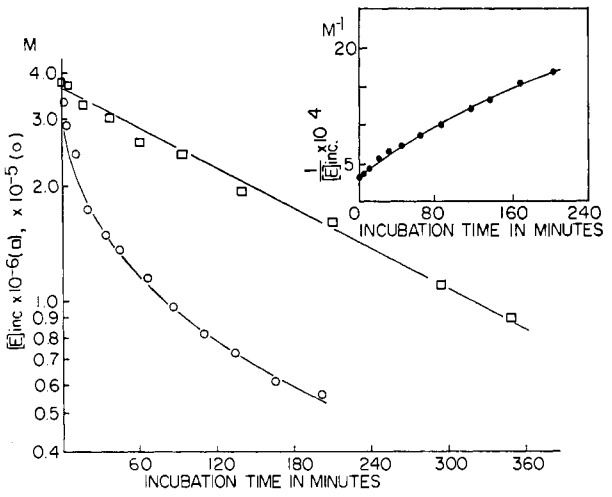

FIGURE 6: Inactivation of α-chymotrypsin at pH 7.9, 40°; semilogarithmic plot of rate of inactivation at two initial concentrations, ~1.0 mg/ml (○) and ~0.1 mg/ml (□). Incubation was in 0.1 M Tris-HCl (ionic strength ~0.1). Aliquots (20–100 μl) were withdrawn at various time intervals and assayed with *N*-acetyl-L-tyrosine ethyl ester at pH 7.0, 30°. The concentration of active enzyme remaining was calculated from [E]assay, assuming $k_0 = 130\ sec^{-1}$. Figure 6 inset is a second-order plot of the data at pH 7.9, 40° at enzyme concentration of ~1.0 mg/ml.

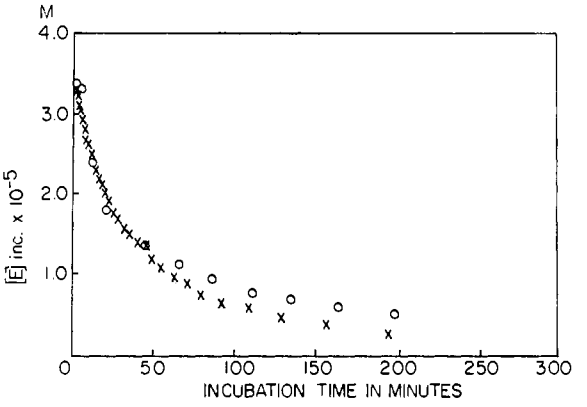

FIGURE 7: A progress curve for the autolysis of α-chymotrypsin at pH 7.9, 40°, 0.1 M Tris-HCl, and ionic strength of ~0.1. Enzyme concentration was ~1.0 mg/ml. (○) represents the experimental points, and (×) the computer-calculated curve based on eq 6. The values of $k_1K_1K_2[H^+]$ and $k_2K_1K_2/K[H^+]$ used were $1.4 \times 10^{-3}\ min^{-1}$ and $9.0 \times 10^2\ M^{-1}min^{-1}$, respectively, and were obtained from data in Figure 6.

tical conditions. The increase in base uptake was found to be directly proportional to the loss of active enzyme only up to about 50% inactivation, after which deviations from linearity were observed. The concentration of kinetically active enzyme decreased at a faster rate than the increase in base uptake. The above results indicate that at pH 7.9 and 40° a process not requiring autolysis is also involved in the inactivation of the enzyme.

Discussion

Interpretation of Kinetic Data. Any single model for the autolysis of α-chymotrypsin must meet the following set of conditions: (a) observed second-order kinetics, *i.e.*, dependence on $[E]^2$, under mild conditions (room temperature, pH range 4–10, and concentrations of 1.0 mg/ml or less); (b) observed mixed, first- and second-order, kinetics at higher temperatures and under more extreme conditions; (c) pH dependence of the rate; and (d) consistent with information obtained from other sources about the behaviour of α-chymotrypsin.

The model presented below meets all these conditions, and may be summarized as follows: (a) An equilibrium exists between active and inactive conformations of the enzyme—at all pH values. A simple representation of such a model showing the various ionization equilibria (Bender *et al.*, 1966; Himoe *et al.*, 1967) is

$$\begin{array}{ccccc} E_iH & \underset{K_3}{\rightleftharpoons} & E_i + H^+ & \xrightarrow{k_1} & E_z \\ {\scriptstyle K_4}\uparrow\downarrow & & & & {\scriptstyle K_2}\uparrow\downarrow \\ EH & \underset{K_1}{\rightleftharpoons} & E + H^+ & & \end{array} \qquad (1)$$

(b) A second step in the autolysis is the formation of a complex between active and inactive conformations of the enzyme.

$$EH + E_i \underset{K}{\rightleftharpoons} [EH \cdot E_i] \xrightarrow{k_2} EH + \text{products} \qquad (2)$$

Thus both the conformational equilibrium and the equilibrium pertaining to the formation of the complex between active and inactive enzyme control the rate of autolysis.

Under conditions of high temperature (about 40°), of pH values greater than 10, or in which denaturing agents like urea and guanidine hydrochloride are present, the above equilibria are disturbed by the unimolecular step which also leads to the formation of a denatured enzyme.

The observed inactivation of the enzyme is, therefore, the sum of two processes, a *first-order* denaturation of E_i and the *second-order* digestion of E_i by native active enzyme, EH, or

$$v = k_1[E_i] + k_2[EH \cdot E_i] \qquad (3)$$

At relatively low temperatures only the second term of the equation is significant; using eq 1 and 2 this can be written as

$$v = \frac{k_2K_1K_2}{K[H^+]} \times [EH]^2$$

or

$$v = \frac{k_2K_3K_4}{K[H^+]} \times [EH]^2 \qquad (4)$$

since $K_1K_2 = K_3K_4$, assuming denaturation is slow compared with protonation; and the rate can be expressed as

$$v = k_{app} \times [EH]^2 \qquad (5)$$

where $k_{app} = k_2K_1K_2/K[H^+]$ or $k_2K_3K_4/K[H^+]$.

At high temperatures (about 40°) both terms of eq 3

become significant, and the equation for inactivation of the enzyme can be written as

$$v = \frac{k_1K_1K_2}{[H^+]} \times [EH] + \frac{k_2K_1K_2}{K[H^+]} \times [EH]^2 \qquad (6)$$

Since both of the processes produce inactive materials, they are indistinguishable in terms of change in kinetic parameters of the enzyme.

Scheme for Autolysis. That the mechanism of autolysis involves an attack of one enzyme molecule on another is not a new concept, and is well documented in the process of "zymogen activation," where a specific cleavage results in the production of active enzyme. Studies of zymogens which can be cited are, for instance, the pseudo-unimolecular kinetics for activation of trypsinogen by Bates and Koch (1935) and Northrop *et al.* (1948), of pepsinogen by Herriott (1938), and of chymotrypsinogen by Kunitz and Northrop (1935) and Jacobson (1947), where the zymogen is present in large excess. A similar (though stepwise) activation is known to produce the enzymes of the chymotrypsin family (Desneulle, 1960).

Consistent with the mechanism for autolysis of chymotrypsin proposed in this communication is the study of trypsin inactivation by Kunitz and Northrop in 1934. The results showed that the course of inactivation between pH values of 2 and 9 follows bimolecular kinetics. Between pH values of 10 and 12, mixed kinetics (first and second order) were observed. They also showed that the equilibrium between the native and denatured forms of the trypsin was dependent upon temperature.

This scheme for autolysis differs from the mechanisms proposed by Bender *et al.* in 1964, by Erlanger *et al.* in 1964 and by Hofstee in 1965. Bender *et al.* (1964) proposed that the inactivation of chymotrypsin is a first-order reaction. This interpretation was based upon active-site titrations (up to ~50% inactivation of the enzyme—at which precipitation occurred). High enzyme concentrations (*ca.* 2–27 mg/ml) were employed. It is apparent from our studies that the observed kinetics of denaturation change at higher enzyme concentrations. It is probable that self-polymerization of the enzyme occurred (Rao and Kegeles, 1958; Martin and Niemann, 1958), making any interpretation of the mechanism very difficult. At the highest concentration (1.0 mg/ml) employed in the present experiments the enzyme exists almost completely in a monomeric state (Rao and Kegeles, 1958). Other results (S. Kumar, unpublished data) obtained at high enzyme concentrations (10 mg/ml or higher; where appreciable amounts of dimers and trimers are presumably present) and at temperatures higher than 40° showed that the kinetics of inactivation are quite complex. Thus it appears that the present results do not correspond to the inactivation of a system containing monomers and polymers. In agreement with the scheme proposed for autolysis, second-order kinetics were also observed at a lower enzyme concentration (0.4 mg/ml) at pH 9.15, 30°.

Erlanger *et al.* (1964) showed their enzyme preparation contained two active components with distinctly different specific activities, heat stabilities, and electrophoretic mobilities. Inactivation of the enzyme occurred by two simultaneous first-order processes. The experiments were performed at only one enzyme concentration (about 4 mg/ml). In our study (see Figure 6) two different protein concentrations (0.1 and 1.0 mg per ml) were used. Different initial slopes but similar final slopes are observed—an unlikely result if there were two separate species. It should, however, be pointed out that the present analysis does not actually exclude the existence of two active forms of the enzyme; but it shows that these forms are kinetically alike within the limits of the experimental method.

The experiments of Hofstee (1965) are primarily based upon the autolysis in the initial 5–20 min, and were done at low ionic strength. Hofstee interpreted his results from the viewpoint that a common rate-limiting process of denaturation exists, since the spectral and activity changes on urea denaturation of chymotrypsin (Chervenka, 1958) were similar to those observed in autolysis. The existence of such similarities may be just a coincidence. In the present experiments, autolysis was followed for extended periods of time (about 75% consumption of the enzyme) and at relatively higher ionic strength, which gives a description of the autolysis process more in line with the typical studies using chymotrypsin. Hofstee's results also showed 35 and 50% inactivation of the enzyme at pH 8.6 and 9.1, respectively, during the initial 15 min of autolysis. Such unusually high inactivation rates were not obtained under our conditions.

Our results can be reconciled with those of other investigators if we recognize that the two complex observed rate constants, k_1 and k_2, show very different dependencies on temperature, ionic strength, and other experimental factors. By carrying out our denaturation experiments under mild conditions of concentration, pH, and ionic strength, we were able to find a range of conditions in which the second-order term predominated. At extreme pH values and at temperatures higher than 40°, we obtained results similar to those of other investigators: either complex kinetic behavior or the predominance of the first-order term.

Consistent with the data of Wu and Laskowski (1956), no evidence for the existence of stable active fragments during autolysis was found by the present authors. Such active fragments, if present, have only transitory existence, not detectable by our method.

The pH Dependence of Autolysis. In the proposed model for the mechanism of α-chymotrypsin denaturation (eq 5), the apparent second-order rate constant (k_{app}) at any pH value is a complex function of the constants k_2, K_1, K_2, K, and $[H^+]$. An overall conformational equilibrium (partially described by K_1 and K_2) can be assigned a pK_{app} value of about 9. The dependence of k_2 and K on pH is not known. However, if the variation of the rate and equilibrium constants for the specific amide substrates can be applied to the process of autolysis (Bender *et al.*, 1966; Himoe *et al.*, 1967), then the dependence of the apparent second-order rate on the hydrogen ion concentration may be described.

At pH values of up to about 8 most of the enzyme is present in the (active) conformation EH (eq 1). Therefore the concentration of protein substrate (inactive conformation) available is almost negligible, and so the autolytic rate is insignificant. As the pH increases above 8, the concentration of the protein substrate increases; also, the autolysis rate increases, reaching a maximum value at about pH 9.0, where the protein substrate and enzyme are present in roughly equal concentrations. As the pH is increased further, two processes occur. First, more of the enzyme is present in the inactive rather than

active conformation. Secondly, the dissociation constant (K) for the active enzyme–protein substrate complex increases. Therefore the rate of autolysis, which is dependent upon both the overall pK_{app} of the conformational change and the pK_{app} of the complex, decreases. The sharpness of the pH optimum (Figure 4) suggests that these two pK_{app} values are very similar.

Conclusion

In conclusion, it may be emphasized that the results based on the kinetics of autolysis (Figures 1–4), on the appearance of ninhydrin-positive species (Figure 5) and on the base uptake at constant pH, clearly suggest the following: (a) α-chymotrypsin used in this study does not appear to be a mixture of two kinetically distinguishable species; (b) autodigestion is chemically distinguishable from the process of denaturation; (c) no enzymatically active intermediate-size fragments are detectable within the time limits of the experiments; (d) the mechanism of autolysis is not first order: it is much more complex, but it can be simplified to represent an essentially second-order process; and (e) the process of autodigestion proceeds either by a "one-by-one" (Tiselius and Eriksson-Quensel, 1939) or by a modified "one-by-one" process (Linderstrøm-Lang, 1950), where the rate-determining step is the initial attack of the enzyme molecule on the protein substrate. This is followed by a fast degradative step(s).

It seems likely, too, that the mechanism of autolysis of endopeptidases is related to the mechanism of zymogen activation.

Reference

Bates, R. W., and Koch, E. C. (1935), *J. Biol. Chem. 111*, 197.

Bender, M. L., Clement, G. E., Kezdy, F., and Heck, H. d'A. (1964), *J. Am. Chem. Soc. 86*, 3682.

Bender, M. L., Gibian, M. J., and Whelan, D. J. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 833.

Chernikov, M. P. (1955), *Biokhimiya 20*, 657.

Chervenka, C. H. (1958), *Biochim. Biophys. Acta 31*, 85.

Cunningham, L. W., Jr., and Neurath, H. (1953), *Biochim Biophys. Acta 11*, 310.

Desneulle, P. (1960), *Enzymes 4*, 107.

Erlanger, B. F., Cooper, A. G., and Bendich, A. J. (1964), *Biochemistry 3*, 1880.

Ginsberg, A., and Schachman, H. K. (1960), *J. Biol. Chem. 235*, 115.

Haehn, H., and Leopold, H. (1934), *Wochschr. Brau 51*, 353.

Herriott, R. M. (1938), *J. Gen. Physiol. 22*, 65.

Herriott, R. M., Desreux, V., and Northrop, J. H. (1940), *J. Gen. Physiol. 24*, 213.

Herriott, R. M., and Northrop, J. H. (1934), *J. Gen. Physiol. 18*, 35.

Himoe, A., Parks, P. C., and Hess, G. P. (1967), *J. Biol. Chem. 242*, 919.

Hofstee, B. H. J. (1965), *Arch. Biochem. Biophys. 112*, 224.

Jacobson, C. F. (1947), *Compt. Rend. Trav. Lab. Carlsberg Ser. Chim. 25*, 325.

Kumar, S., and Hein, G. E. (1969), *Anal. Biochem. 30*, 203.

Kunitz, M., and Northrop, J. H. (1934), *J. Gen. Physiol. 17*, 591.

Kunitz, M., and Northrop, J. H. (1935), *J. Gen. Physiol. 18*, 433.

Linderstrøm-Lang, K. (1950), *Cold Spring Harbor Symp. Quant. Biol. 14*, 117.

Martin, R. B., and Niemann, C. (1958), *J. Am. Chem. Soc. 80*, 1473.

Northrop, J. H., Kunitz, M., and Herriott, R. (1948), Crystalline Enzymes, 2nd ed, New York, N. Y., Columbia University.

Perlman, G. E. (1954), *Nature 173*, 406.

Rao, M. S. N., and Kegeles, G. (1958), *J. Am. Chem. Soc. 80*, 5724.

Rosen, H. (1957), *Arch. Biochem. Biophys. 67*, 10.

Tiselius, A., and Eriksson-Quensel, I. B. (1939), *Biochem. J. 33*, 1752.

Wainfan, E., and Hess, G. P. (1960), *J. Am. Chem. Soc. 82*, 2069.

Wells, G. H., and Benson, R. L. (1907), *J. Biol. Chem. 3*, 35.

Wu, F. C., and Laskowski, M., Jr. (1956), *Biochim. Biophys. Acta 19*, 110.