Two Steps in the Reaction of Chymotrypsin with Acetyl-L-Phenylalanine Ethyl Ester

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Studies of the effect of the hydrogen-ion concentration on the catalytic activity of various enzymes have long been recognized as a powerful tool for the exploration of the nature of the active sites as well as of the mechanisms involved in such reactions (Dixon, 1953; Alberty, 1954; Alberty, Massey, Frieden & Fuhlbridge, 1954; Wilson, 1954). One of us (Gutfreund, 1955) has found that the ionization constant of the catalytic site of the enzyme could be determined accurately for the trypsin-catalysed hydrolysis of benzoyl-L-arginine ethyl ester. Moreover, owing to a number of fortunate properties of that system, there is less ambiguity about the properties and chemical nature of the catalytic site of trypsin than there is about other enzymes which have been studied in a similar way. However, neither in the case of trypsin nor in that of the chymotrypsin system to be described here is the chemical nature of the ionizing catalytic group identified beyond any doubt, though one can say that the imidazole group of histidine would have all the necessary properties. Apart from a study of the catalytic site the present investigation was undertaken to compare the reaction mechanism of chymotrypsin with that of trypsin. For trypsin Gutfreund (1955) found that the reaction of the enzyme with its substrate involves two separate steps: first, an initial fast adsorption at the specificity site; and secondly, a slow, rate-determining step involving a reaction of the catalytic site with the carbonyl groups of the substrate. In the trypsin-catalysed reaction the enzyme–substrate combination was shown to be independent of the subsequent reaction at the catalytic site and similar conclusions can be drawn from the results of experiments with chymotrypsin to be described here. Earlier work on the chymotrypsin-catalysed hydrolysis of derivatives of phenylalanine and tryosine has been reviewed by Neurath & Schwert (1950).

EXPERIMENTAL

The procedure for following the rate of ester hydrolysis used in this laboratory has been described recently (Bernhard, 1955). One minor modification was the use of a magnetic stirrer. Instead of bubbling nitrogen through the reaction mixture it was circulated in the space above it. Under these conditions CO_2-free water did not change its pH during prolonged stirring. All reactions were carried out in a water bath maintained at $25 \pm 0.05°$.

Crystalline α-chymotrypsin was obtained from Worthington Biochemicals Corporation, Freehold, N.J. For each day's experiments an enzyme solution was made up by 500-fold dilution in water from a single stock solution containing about 0·5% enzyme protein in 0·1 M-NaH_2PO_4–Na_2HPO_4 buffer at pH 5·0. One ml. of this enzyme solution was added to 14 ml. of a solution of appropriate pH, buffer and substrate concentration. The reaction mixture was 0·1 M with respect to NaCl.

The substrate, acetyl-L-phenylalanine ethyl ester, was the same sample used and described by Bernhard (1955).

It was found that tenfold changes in the concentration of three buffer systems (0·005–0·05 M) used (diethylamine, glycine and ethylenediaminetetraacetic acid) had no inhibitory or accelerating effect on the hydrolysis reaction catalysed by chymotrypsin, and thereafter glycine was used as buffer at pH 8 and ethylenediaminetetraacetic acid at all other pH values.

RESULTS

The reaction scheme:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

(where E, S, ES and P are the enzyme, substrate, enzyme–substrate complex and product respectively) leads to the kinetic expression for the velocity v,

$$v = k_3[E][S]/(K_m + [S]) \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

The results are expressed in the form of the pH dependence of K_m and of v_0, where v_0 is the initial velocity at concentration of substrate $[S_0]$. Values for K_m were obtained by plotting $1/v_0$ against $1/[S_0]$ (Lineweaver & Burk, 1934), the value of $1/[S_0]$ at $1/v_0 = 0$ being equal to $-1/K_m$, as shown in Fig. 1 for measurements on the system chymotrypsin–acetyl-L-phenylalanine ethyl ester at pH 7.

The values for K_m over the range of pH 6·5–9 are given in Fig. 2 and a plot of $k_3[E]$ against pH is given in Fig. 3; $k_3[E]$ was calculated from experiments at $[S_0] = 1.97 \times 10^{-3}$ M by Eqn. 1. $k_3[E]$ is in arbitrary units, since only relative values are required for our conclusions. A calculation of the absolute rate of hydrolysis of acetyl-L-phenyl-

alanine ethyl ester at 25° and pH 7·8, based on the molecular weight of chymotrypsin $M = 23\,000$, gives the value $k_3 = 160$ sec.$^{-1}$.

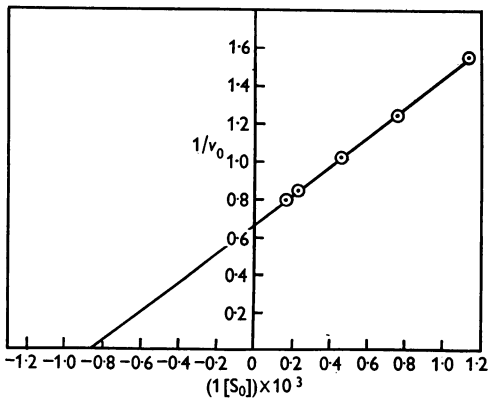

Fig. 1. Determination of K_m for the reaction of chymotrypsin with acetyl L-phenylalanine ethyl ester at pH 7 and 25°. K_m and the initial substrate concentration $[S_0]$, are in moles/l. The initial velocity, v_0, is in arbitrary units.

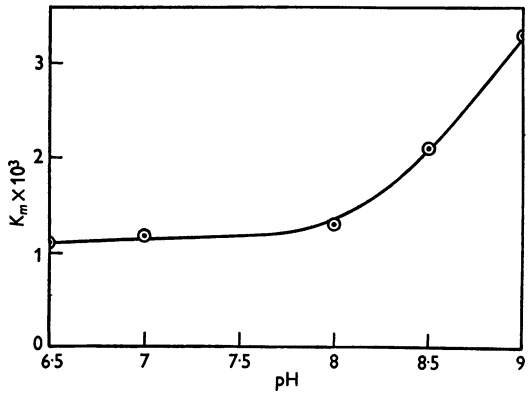

Fig. 2. Variation of K_m with pH for the reaction between chymotrypsin and acetyl L-phenylalanine ethyl ester at 25°. K_m is in moles/l.

DISCUSSION

The Michaelis constant $K_m = (k_2 + k_3)/k_1$ as defined by Briggs & Haldane (1925) approximates to the true equilibrium constant $K_m = [E][S]/[ES]$ if $k_3 \ll k_2$. Gutfreund (1954) has pointed out that for the chymotrypsin-catalysed hydrolysis of acetyl-L-phenylalanine ethyl ester k_3 is small compared with k_2. The results reported here, which show that K_m is constant from pH 6·5 to 8·0, while $k_3[E]$ changes fourfold over this pH range, are consistent with K_m being the true equilibrium constant of enzyme–substrate binding.

The Michaelis–Menten equation, which involves only one form of the enzyme–substrate complex, is an over-simplification but it is adequate for the derivation of the kinetics for the formation and decomposition of the rate-determining complex. It follows from the results of the effect of pH on the maximum velocity $k_3[E]$ that hydrogen ions act as non-competitive inhibitors of chymotrypsin-catalysed hydrolysis reactions. An analytical description of the general principle of such inhibitions has been given by Botts & Morales (1953). One can conclude from this that the reaction at the specific

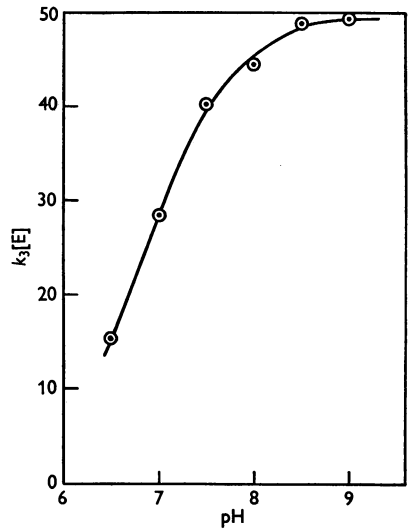

Fig. 3. Variation of the maximum velocity, $k_3[E]$ (in arbitrary units) with pH for the reaction between chymotrypsin and acetyl L-phenylalanine ethyl ester at 25°.

site of the enzyme, resulting in the formation of the initial enzyme–substrate complex characterized by K_m, is independent of the subsequent reaction of the substrate with the catalytic site of the enzyme. We can therefore expand the Michaelis–Menten equation as follows

$$E + S \underset{}{\overset{k_1}{\rightleftharpoons}} (ES)_1 \overset{k_3}{\rightleftharpoons} (ES)_2 \rightarrow E + P.$$

The rate of enzyme–substrate complex formation is very large, $k_1 > 10^6$ l. M^{-1} sec.$^{-1}$ (Gutfreund, 1954), while k_3, the rate-determining formation of a labile chemical intermediate compound $(ES)_2$, is 100–200 sec.$^{-1}$, for various chymotrypsin preparations. A more detailed picture as well as the analogy of this second step with the nucleophilic attack of an electron donor on the carbonyl carbon during base-catalysed ester hydrolyses has been given by Gutfreund (1955).

The ionization constant of the catalytic site can be determined either by the method used by

Gutfreund (1955) or by the determination of K_i, the inhibition constant,

$$K_i = [E][H^+]/[EH^+],$$

where [E] is the concentration of active enzyme and $[EH^+]$ the concentration of inhibited enzyme. From the equations given by Lineweaver & Burk (1934) one can get the following relation between inhibitor concentration $[H^+]$, $k_3[E]$ the maximum velocity at a particular $[H^+]$ concentration and $k_3[E_0]$ the maximum velocity when all enzyme present is in the active form

$$[E_0]/[E] = 1 + [H^+]/K_i.$$

The ratio $[E_0]/[E]$ at different values of $[H^+]$ was calculated from determinations of $k_3[E]$, taking $k_3[E_0] = k_3[E]$ at pH 9·0. The dissociation constant $K_i = 1{\cdot}42 \times 10^{-7}$ and $pK_i = 6{\cdot}85$ for the catalytic site of chymotrypsin has been calculated from the slope of the plot of $[H^+]$ against $[E_0]/[E]$ given in Fig. 4. In the case of trypsin hydrolysing benzoyl-L-arginine ethyl ester pK_i (= 6·25) was found to be constant in the presence of phosphate, *p*-nitrophenol and ethylenediaminetetraacetic acid as buffer (unpublished observations). Sri Ram, Terminiello, Bier & Nord (1954) have studied the pH dependence of the casein hydrolysis by trypsin and by acetylated trypsin. Approximate pK_i values can be determined from their data and come to 6·25 for trypsin, a very basic protein, and 7·10 for acetyltrypsin, an acidic protein. Such a shift of the pK of the catalytic site of the acetylated enzyme to a more alkaline pH is to be expected. Similarly, since chymotrypsin is a less basic protein than trypsin, the pK of the catalytic site should be at a more alkaline pH in the case of chymotrypsin even if the sites are chemically identical in the two enzymes. Further evidence for the identification of the imidazole group of histidine as the catalytic site of chymotrypsin was summarized by Desnuelle (1954). Model reactions of imidazole derivatives were studied by Wagner-Jauregg & Hackley (1953) and the inactivation of chymotrypsin during photo-oxidation was described by Weil & Buchert (1952). Though all the evidence as to the chemical nature of the catalytic group is necessarily only circumstantial, the known physical properties and chemical reactivity of imidazole would account for the behaviour of the catalytic site.

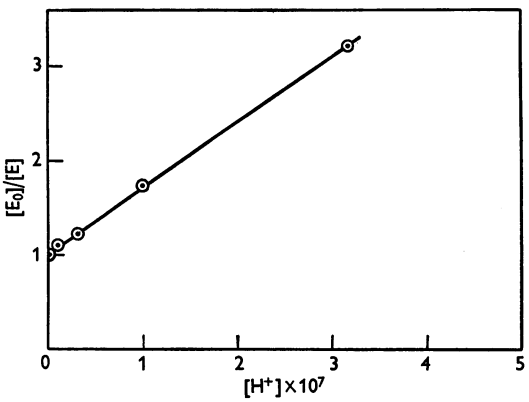

Fig. 4. Determination of the inhibition constant, K_i (in moles/l.) for the non-competitive inhibition of chymotrypsin by hydrogen ions. The slope of the graph is $1/K_i$.

An acyl migration from histidine to serine might form part of the over-all reaction (Desnuelle, 1954). The above results would be consistent with such an additional step, provided that all steps taking place after the formation of $(ES)_2$ are fast compared with the rate of formation of $(ES)_2$.

SUMMARY

1. The effect of pH on k_3, the first-order rate constant for the decomposition of the enzyme–substrate complex, and on K_m, the Michaelis constant, has been studied for the chymotrypsin-catalysed hydrolysis of acetyl-L-phenylalanine ethyl ester.

2. The results presented are consistent with our previous finding that K_m is the true dissociation constant $E + S \rightleftharpoons ES$ and that this initial rapid enzyme substrate combination is independent from the subsequent catalytic step.

3. Evidence for the chemical nature of the catalytic site of chymotrypsin is summarized and it is suggested that it is the imidazole group of histidine.

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