KINETICS OF CHYMOTRYPSIN WITH A HOMOLOGOUS SERIES OF *n*-FATTY ACID ESTERS AS SUBSTRATES*

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SUMMARY

An investigation has been made of the hydrolysis by chymotrypsin of *n*-fatty acid esters of *ortho*- and *meta*-hydroxybenzoic acids at pH values (6.8–8.3) where these compounds are soluble and completely ionized. The evidence indicates that, on the basis of consecutive acylation and deacylation reactions, the hydrolysis can be described by $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E +$ products, where ES is the "Michaelis" complex and k_2 the constant for acylation of the enzyme that is rate limiting for the overall reaction.

The influence of pH on the rate revealed uncompetitive inhibition by hydrogen ions indicating that the pK_H of ES is considerably higher than that of the free enzyme. From variation of V_m with pH the pK_H values for the *ortho*- and *meta*-esters were found to be 7.55 and 7.25 respectively but on the basis of $V_m = k_2E_t/(1 + H/K_H)$ the k_2-values are about the same for the two types of substrate.

The constant k_2 increases exponentially with the acyl C-chain length of the substrate up to C_7 and decreases sharply thereafter. This pattern is determined only by the acyl moiety of the substrate. The ratios of the Michaelis constants for C_5, C_6, C_7, C_8 and C_9, which appear to approximate equilibrium constants (k_{-1}/k_1), are about 4:4:2:1:1.

INTRODUCTION

Previous results have shown that chymotrypsin hydrolyzes *n*-fatty acid esters of *meta*-hydroxybenzoic acid[1]. The maximal initial reaction rate (V_m) increases exponentially with increasing acyl C-chain length up to C_7. Since the nonenzymic hydrolysis of these substrates is not influenced by the C-chain length from C_4 on[2], it would seem that this observation reflects an essential difference between enzymic and non-enzymic catalysis. Furthermore, since the experimental kinetic constants (V_m, K_M) of different esterolytic enzymes display characteristic patterns with respect to the C-chain length of the substrates[1–4], subtle differences in structure of the active sites of the enzymes might be revealed. However, a prerequisite to such studies is the interpretation of the experimentally determined (apparent) kinetic constants in

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terms of the elementary constants of the underlying single steps that make up the overall reaction.

In view of recent findings that esters act as acylating agents for chymotrypsin[5], the enzymic reaction could be written as

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E\text{-}P_2 + P_1 \tag{1}$$

$$E\text{-}P_2 + H_2O \xrightarrow{k_3} E + P_2, \tag{1a}$$

where S is the substrate, E the free enzyme, ES the "Michaelis" complex, E-P_2 the intermediate acylated enzyme, and P_1 and P_2 the two products. The same symbols will be used to denote concentrations. It is found that the relationship between the overall initial rate (v) and S is given by the original Michaelis equation $v = V_m/(1 + K_M/S)$

where
$$V_m = E_t/(1/k_2 + 1/k_3) \tag{2}$$

and
$$K_M = (k_{-1} + k_2)/k_1 \cdot (1 + k_2/k_3) \tag{2a}$$

Identical expressions were obtained by GUTFREUND AND STURTEVANT[6]. The equations refer to a steady state situation whereby the rates of formation of the two products are equal and the concentrations of E, ES and E-P_2 are constant. They apply only to initial reaction rates whereby the reverse of the reactions represented by k_2 (acylation constant) and k_3 (deacylation constant) can be neglected.

The activity of the enzyme seems to depend on the presence of a deprotonated imidazole group[5] and it has been shown[6,7] that the influence of pH on the rate of both acylation and deacylation can be described as a reversible inhibition by protons, which combine in a 1:1 ratio with the active center of the enzyme. Without further assumptions such a mechanism results in a pH maximum rather than a pH optimum*.

On this basis the constants k_2 and k_3 in the above equations are the "true" constants referring to a pH at which the pertaining group in E, ES, and E-P_2 is completely deprotonated. It is found[6,8] that at lower pH, where these enzyme species are partly proton-bound and inactive, the apparent constants are given by

$$k_2' = k_2/(1 + H/K_{H_2}) \tag{3}$$

$$k_3' = k_3/(1 + H/K_{H_3}) \tag{3a}$$

where H is the hydrogen ion concentration and K_{H_2} and K_{H_3} are the hydrogen ion dissociation constants of HES and HE-P_2 respectively.

Even when the same proton-accepting group is involved in acylation and in deacylation, a different influence of pH on k_2' and k_3' may be expected because covalent bond formation in the reaction $ES \longrightarrow E$-P_2 might change the electron distribution in the active center and thus affect its affinity for hydrogen ions. For this reason K_{H_2} and K_{H_3} do not necessarily have the same value[6].

When the substrate carries a charge it may be assumed that the pH also influences the apparent constants k_1' and k_{-1}' (see below).

In the present investigation the influence of pH on V_m and K_M of chymotrypsin

* An optimum could nevertheless occur, *e.g.* through hydrogen ion dissociation of a group in the active center other than the primary one in a pH region where the rates of acylation and of deacylation are already maximal.

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with *o*-heptanoyloxybenzoic acid as the substrate has been determined. From these and other data the relative magnitude of the underlying elementary constants has been estimated on the basis of the above equations. The results have been applied to establish the influence of the *n*-acyl-C-chain length of the substrate on some of these constants.

EXPERIMENTAL

Enzyme, substrates, assay

Recrystallized chymotrypsin (presumably the "*a*"-form) was the same preparation as used previously[1]. However, the substrates were the *n*-fatty acid esters of *ortho*- as well as of *meta*-hydroxybenzoic acid because the Michaelis constants with the latter substrates are too small to be measured accurately even with the present assay procedure. Those of the *ortho*-esters are of the order of 30 times as large[1]. The substrates are referred to as *o*-C_n and *m*-C_n according to the number (n) of C-atoms in the acyl moiety. They are completely ionized and soluble in the pH region (6.8–8.3) that was applied.

Details of the assay procedure have been described previously[1,9,10]. It is based on continuous spectrophotometric measurement of the liberated hydroxybenzoic acid, directly in the reaction mixture. The rate determinations were carried out at 29° in 0.01–0.037 *M* Veronal buffer. It was found that the buffer, in this concentration range, had a small activating effect noticeable only at low substrate concentrations. Frequent use was made of a cuvette with seven compartments and a corresponding positioning rod (Courtesy of Pyrocell Company, New York) adapted to the Beckman U.V. spectrophotometer Model DU. This accessory allowed six rate determinations to be carried out simultaneously.

RESULTS

Influence of pH on V_m and K_M

Fig. 1a shows the influence of pH on the activity (v) at different concentrations (S) of the substrate *o*-C_7. Based on the equation $V_m = v + K_M \cdot v/S$ these data, plotted as v versus v/S at a fixed pH, yield straight lines with negative slopes equal to K_M. The intercept with the ordinate ($S \longrightarrow \infty$) is V_m, while the intercept with the abscissa ($S \longrightarrow 0$) represents V_m/K_M[11–13].

In Fig. 1b the V_m-values are plotted as V_m versus $V_m \cdot H$[8]. The intercept with the ordinate gives V_m for $H \longrightarrow 0$, while the reciprocal slope represents the corresponding K_H. The V_m-values for the *meta*-C_7 ester were determined at a substrate concentration of 10^{-2} *M*. Since in this case K_M is of the order of 10^{-4} *M* at pH 8[1], and like that of *o*-C_7 (Fig. 1a) may be assumed to decrease with decreasing pH, the enzyme is practically saturated with substrate at this concentration.

The first task in the interpretation of these data is to determine the relative magnitude of the constants k_2 and k_3 in eqns. (2) and (2a) and to establish which, if any, is rate limiting. The following evidence indicates that the overall rate of the enzymatic reaction is limited by the acylation reaction $ES \xrightarrow{k_2} E\text{-}P_2 + P_1$.

1. If k_2' and k_3' were of the same order of magnitude one could not expect linearity of the curves in Fig. 1b, where $V_m = E_t/(1/k_2' + 1/k_3')$ because the rates of acylation and deacylation are influenced differently by the pH (see INTRODUCTION).

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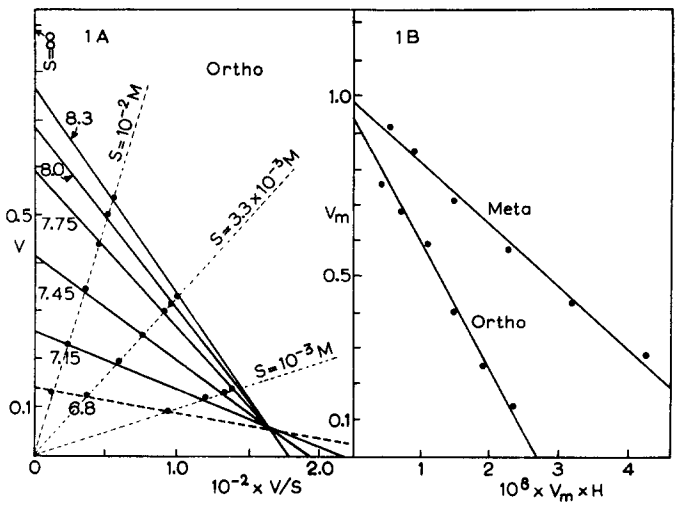

Fig. 1A. Influence of the concentration (S) of the substrate (o-C_7) on the initial reaction rate (v, μmoles/3 ml/30 min/0.2 mg enzyme) in 0.01 M Veronal buffer of different pH (indicated on the curves). The intercepts of the v versus v/S plots with the ordinate show the influence of pH on V_m. The slopes represent K_M-values.

Fig. 1B. Influence of the hydrogen ion concentration (H) on V_m of o-C_7 (*ortho*) and of m-C_7 (*meta*) plotted as V_m versus $V_m \cdot H$. The data for o-C_7 are derived from Fig. 1a, those for m-C_7 from rate determinations at 10^{-2} M substrate concentration[1]. The intercepts with the ordinate are the V_m-values when a pertinent group in the enzyme is completely deprotonated. The reciprocal slopes are the corresponding hydrogen ion dissociation constants[8].

The observed linear relationships would suggest that V_m equals either $k_2E_t/(1 + H/K_{H_2})$ or $k_3E_t/(1 + H/K_{H_3})$.

2. If the hydrolysis of the intermediate acylated enzyme were the rate-limiting step ($k_3' \ll k_2'$) the maximal rate ($V_m = k_3' \cdot E_t$), regardless of pH, should be the same for all esters of a particular fatty acid, since the same intermediate enzyme-ester would be involved. This is clearly not the case with the heptanoyl esters of *ortho*- and *meta*-hydroxybenzoic acids as is seen in Fig. 1b, where the two V_m-curves do not coincide.

3. The change of V_m with the hydrogen ion concentration (Fig. 1b) reveals pK_H-values of 7.55 with o-C_7 and 7.25 with m-C_7 at 29° as compared to 6.96 found for the deacylation of acetyl δ-chymotrypsin at 25° in aqueous solution[7]. The C-chain length apparently has no influence on the pK with respect to V_m, since at least with m-C_5 and m-C_7 as the substrates little or no difference was found (see below). The difference in magnitude of the above pK-values thus indicates that V_m of the present substrates does not reflect the rate of hydrolysis of the acylated enzyme. It is hereby assumed that there is little or no difference in the hydrogen ion dissociation constant of the α- and δ-forms of the enzyme[14].

4. The influence of the C-chain length (C_2–C_5) of the acyl moiety of the ester on the rate of deacylation[7] is entirely different from its influence on V_m of the *meta*-esters[1].

These considerations therefore suggest that at all pH-values $k_2' \ll k_3'$ from which it would follow that $V_m \approx k_2' \cdot E_t$ and $K_M \approx (k_{-1}' + k_2')/k_1'$. It would follow also that a "Michaelis" complex is formed before acylation takes place because the de-

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acylation reaction would be rate-limiting for V_m if the acylated enzyme were formed directly through the reaction $E + S \longrightarrow E\text{-}P_2 + P_1$. In fact, the reaction may be written in the customary manner $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P_1 + P_2$. Therefore, the rate equations and graphical procedures for the determination of kinetic constants, such as those presented previously[8], apply to the system.

The results of Fig. 1a show that V_m and K_M change with pH in the same manner. At high pH these constants change by the same factor, as is demonstrated by a constant intercept with the abscissa. This suggests "uncompetitive" inhibition by hydrogen ions[8], *i.e.* in this pH region the hydrogen ions combine with the enzyme–substrate complex but not with the free enzyme. Thus the pK_H of the free enzyme must be much lower than that of *HES*. Theoretically the former could be determined from the intercepts with the abscissa at low pH, but the data are insufficient for an accurate estimation. The high pK_H of *HES* is possibly due to the negative charge on the ionized carboxyl group of the substrate (see DISCUSSION).

As follows from equations for uncompetitive inhibition[8] the apparent Michaelis constant equals $(k_{-1} + k_2)/k_1 \cdot (1 + H/K_{H_2})$. Since $V_m = k_2 \cdot E_t/(1 + H/K_{H_2})$ the common intercept at pH-values around 8 gives $k_1 \cdot k_2 \cdot E_t/(k_{-1} + k_2)$. These relationships do not hold true at lower pH-values where the concentration of the active free enzyme (E) is influenced by the hydrogen ion concentration. Here the intercept with the abscissa (Fig. 1a) increases. This may be due to an increase of the apparent k_1 on account of the positive charge acquired by the active center of the enzyme which increases the affinity for the negatively charged substrate.

Influence of the C-chain length of the substrate on the kinetic constants

Fig. 2 shows the influence of the C-chain length of the n-fatty acid moiety of the *ortho*-esters on the reaction rate (v) at different substrate concentrations (S). Since these determinations were carried out at pH $\approx$ 7.8 it follows from the above con-

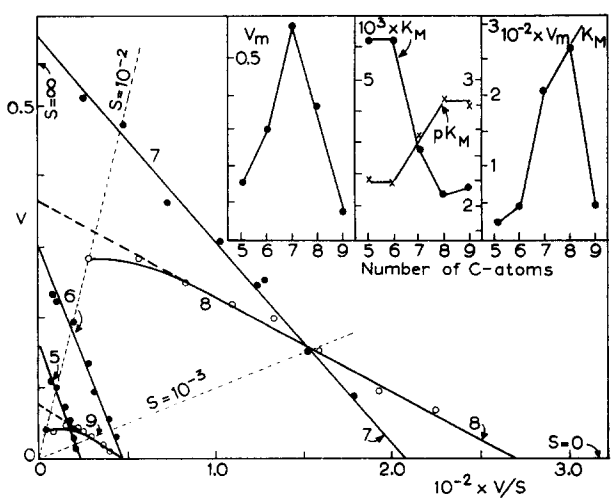

Fig. 2. Plots of v versus v/S with *ortho*-esters of different n-acyl C-chain length (indicated on the curves) in 0.037 M Veronal buffer of pH $\approx$ 7.8. The influence of the C-chain length on the constants V_m, K_M and V_m/K_M, derived from these plots, is shown in the inserts. All data refer to 0.2 mg enzyme, but for C_5 and C_9 a higher enzyme concentration was actually used.

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siderations that V_m (intercept with the ordinate) equals $k_2 \cdot E_t/(1 + H/K_{H_2})$, K_M (slope) is given by $(k_{-1} + k_2)/k_1 \cdot (1 + H/K_{H_2})$ while V_m/K_M (intercept with the abscissa) gives the "true" first order constant[13], independently of pH.

As mentioned above, it was established that K_{H_2} with *m*-C_7 is the same as that with *m*-C_5 as the substrate *i.e.* the C-chain length does not influence the hydrogen ion dissociation of the enzyme–substrate Michaelis complex. Therefore, V_m represents the *relative* k_2-values of the different substrates. The influence of the C-chain length on V_m (insert Fig. 2) is the same as found previously[1] for the *meta*-esters indicating that the k_2-pattern is determined only by the fatty acid moiety of the substrate.

On the same basis K_M is directly proportional to $(k_{-1} + k_2)/k_1$. It may be observed that K_M is approximately the same for C_5 and C_6 and again for C_8 and C_9. Since in each case there is a great difference in k_2 it would appear that $k_2 \ll k_{-1}$. Thus K_M-values would represent the relative dissociation constants of the enzyme–substrate complexes (see insert Fig. 2).

The third insert in Fig. 2 shows the influence of the C-chain length on V_m/K_M that may be looked upon as the constant of the overall acylation reaction $E + S \rightleftharpoons ES \longrightarrow E\text{-}P_2 + P_1$.

DISCUSSION

DIXON AND NEURATH[7] find that the influence of pH on the rate of enzymic hydrolysis of acetyl-L-tyrosine ethyl ester is similar to its influence on the rate of deacylation of acetyl chymotrypsin. This might imply that, in contrast to our results, the hydrolysis of the intermediate enzyme-acetyl-L-tyrosine ester is rate-limiting in the enzymic reaction ($k_{2a} \gg k_{3a}$)*. The authors also find that the rate of deacylation of such aromatic compounds is much larger than that of aliphatic derivatives ($k_{3a} > k_3$). Since in our case $k_3 \gg k_2$ it would follow that $k_{2a} \ggg k_2$, *i.e.* the rate of acylation of the enzyme by the amino acid esters is almost instantaneous compared to the rate of acylation by fatty acid esters. Evidently this is connected with the structure on the enzyme surface that is better adapted to amino acid esters than to fatty acid esters.

The relatively high pK_H of acylation at 29°, 7.55 in the case of *o*-C_7, as compared to 6.2 at 3° for *p*-nitrophenyl acetate[7] might partly be the result of the difference in temperature but could also be connected with the negative charge of the present substrates that through induction in the enzyme–substrate complex might enhance the binding of hydrogen ions. This effect is greater for the *ortho*- than for the *meta*-esters (p*K* 7.25) because, as one might reason, in the former the ionized carboxy group is closer to the ester bond and the center of the reaction. When the pH is

* The apparent contradiction between our results and those of GUTFREUND AND STURTEVANT[6], who find that with *p*-nitro phenyl acetate (NPA) k_3 is rate-limiting, might be explained by a possible competition between water and isopropyl alcohol (20%) used by these authors to increase the solubility of the substrate. Especially when the hydrolysis depends on a "water site" in the enzyme that has greater affinity for the alcohol than for water, a relatively low concentration of the alcohol might result in a decrease in k_3 to such an extent that this constant becomes rate limiting. Another possibility would be that the k_3 with NPA is much smaller than that with longer chain fatty acid esters, while the k_2-values are of the same order of magnitude. However, this is not born out by the results of others[7] on the influence of the C-chain length on k_3, nor by the present results applied to previous data[1] that indicate a great decrease of k_2 with decreasing C-chain length.

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increased this difference between the two cases, as reflected in V_m (Fig. 1b), becomes less and less. At sufficiently high pH, when in both cases all the complex is in the active form ($k_2' = k_2$), the rates are almost the same. This indicates that the difference between the *ortho*- and the *meta*-esters, with respect to k_2', is almost entirely due to such electronic forces and is not the result of steric effects. As might be expected, steric effects would mainly influence the formation of the complex and would be less prominent in subsequent reactions after the complex has been formed. This is further emphasized by the fact that at pH $\approx$ 8 the K_M (k_{-1}/k_1)-values of the *ortho*-esters are of the order of 30 times as large as those of the *meta*-compounds[1].

LUMRY[15] has emphasized the fact that our data on the *meta*-esters[1] show an exponential relationship between V_m and the number (C_2–C_6) of *n*-acyl C-atoms in the substrate. In the case of liver esterase[2] we have reported a similar relationship with respect to these esters (C_2–C_{10}). The factors involved are of the same order but the optimum number of C-atoms is different. Other esterolytic enzymes that were investigated in this respect are two pancreatic esterases[3], serum cholinesterase[4] and trypsin[1]. However, in all these cases, in contrast to the present investigations, the meaning of the experimental constants in terms of the underlying "elementary"* constants has not yet been established.

In the present case the ratios of the k_2 values (V_m) for C_5, C_6, and C_7 are about 1:2:4 while the affinities ($1/K_M$) of C_5, C_6, C_7, C_8 and C_9 for the enzyme are related as 1:1:2:4:4. It is interesting to note that the substrate (C_7) with a pK_M half way between the two "levels" has the highest V_m (see inserts in Fig. 2).

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* These constants, of course, are truly elementary only if the reaction can be described by the simple acylation reactions given in the INTRODUCTION, and does not involve additional steps.