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CHYMOTRYPSIN-CATALYZED REACTIONS OF PHENYL HIPPURATE WITH ALCOHOLS. KINETIC EVIDENCE FOR A BINDING SITE FOR NUCLEOPHILES

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SUMMARY

In chymotrypsin (EC 3.4.4.5)-catalyzed reactions of phenyl hippurate, it was found that added alcohols can increase by 2–3-fold the rate of production of phenol, while decreasing the rate of acid release. The total rates of reaction do not vary linearly but level off, indicating binding of alcohol. On binding, the alcohol functions as a nucleophile for attack on the intermediate hippuryl–chymotrypsin, while inhibiting the attack of water. Pentaerythritol was found to be the most effective alcohol, causing a half maximal increase in rate near a concentration of 0.05 M. From the constant ratio of k_{cat}/K_m, and the close similarity of the binding constant for alcoholysis and the inhibition constant for hydrolysis, it is concluded that pentaerythritol binds to a specific nucleophilic site on the hippuryl–chymotrypsin intermediate and prevents water from attacking. This site is apparently not present on the free enzyme.

INTRODUCTION

It is well established that the hydrolysis of esters catalyzed by α-chymotrypsin (EC 3.4.4.5) proceeds with formation of an intermediate acyl–enzyme[1]. A minimal scheme can be written as follows:

$$\mathrm{RCOOR' + } E \rightleftharpoons \mathrm{RCOOR'} \cdot E \xrightarrow{k_2} \begin{matrix}\mathrm{RCO}E \\ +\mathrm{R'OH}\end{matrix} \xrightarrow{k_3[\mathrm{H_2O}]} \mathrm{RCOOH} + E \qquad (1)$$

An indirect approach to the study of the acyl–enzyme intermediate and its reactivity is by adding to the reaction mixture nucleophiles, which compete with water for the intermediate:

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$$\begin{array}{l} \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \text{RCOOH} + E \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad k_3[H_2O] \nearrow \\ \text{RCOOR}' + E \rightleftharpoons \text{RCOOR}'\cdot E \xrightarrow{k_2} \text{RCO}E \qquad\qquad (2) \\ \qquad\qquad\qquad\qquad\qquad\quad +\text{R}'\text{OH} \searrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad k_4[N] \searrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \text{RCON} + E \end{array}$$

Depending on whether k_2 or k_3 (or both) are rate determining, different patterns in the rates of alcohol and acid release can be observed, as has been discussed[2-4].

The added nucleophiles can react from solution or from a site on the enzyme. It is thought that the added nucleophiles must be binding to the enzyme, since their molar reactivity compared to water is much greater than would be expected from non-enzymic reactions. The question of a specific binding site on the enzyme for the nucleophile water, has not yet been resolved[5,6].

In an effort to shed some light on this problem, we report in the present study the effects of oxygen nucleophiles on the chymotrypsin-catalyzed reactions of phenyl hippurate.

EXPERIMENTAL

Materials

α-Chymotrypsin was from Boehringer: analytical, lyophilized and salt free (A_4). Phenyl hippurate, prepared by the method of Weiss (in ref. 7), (adding 17 g $POCl_3$ to a warm mixture of 25 g hippuric acid and 18 g phenol) had m.p. 104 °C (recrystallized from ethanol). Pentaerythritol (Fluka, purum), was recrystallized from boiling water, m.p. 258–260 °C. Its NMR spectrum in ^2H_2O showed no impurities. All other alcohols and reagents used were commercial products of high purity and used without treatment. Glass-distilled water was used throughout.

Methods

Chymotrypsin was dissolved in 10^{-3} M HCl and the solution kept at 4 °C for up to 2 weeks. Its concentration was determined from its ultraviolet absorption spectrum ($\varepsilon = 5.1 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ at 280 nm)[8].

Total rates were determined by monitoring the release of phenol from phenyl hippurate at 275 nm on a Cary 16K spectrophotometer. Reactions were initiated by adding aliquots of a chymotrypsin stock solution to cuvettes containing 3-ml solutions of phenyl hippurate at the desired concentration. These solutions were prepared immediately before use by adding aliquots of a stock substrate solution in acetonitrile to 0.025 M sodium phosphate buffer at pH 7.0. The chymotrypsin concentration was $7.3 \cdot 10^{-7}$ M. The observed rates in absorbance units/min were converted into molar rates by using $\Delta\varepsilon = 1.1 \cdot 10^3$ at 275 nm, which was determined by measuring the difference in absorption between a mixture of phenol–hippurate anion and phenyl hippurate.

Rates of hydrolysis were measured on a Radiometer pH-stat which consisted of the SR2c/ABU 12/TTT 11b assembly with a pH-meter 26. The chymotrypsin concentration was $2.2 \cdot 10^{-6}$ M. The rates were obtained by titrating the release of protons

at pH 7.0 with 0.02 M NaOH. Blank rates of CO_2 uptake (which were usually less than 10% of the observed rate) were accounted for.

In all reaction solutions the ionic strength was kept constant at 0.1 with NaCl. The acetonitrile concentration was 3.3%.

All kinetic parameters were obtained by applying a least-squares treatment to the data.

RESULTS

The rate of phenol release in the chymotrypsin-catalyzed hydrolysis of phenyl hippurate was measured in the presence of various alcohols (Fig. 1). It was found that for many alcohols studied, this rate increased markedly; at alcohol concentrations less than 0.6 M, the rate increased at least 2-fold. Polyhydric alcohols (pentaerythritol, ribitol, glycerol, ethylene glycol and 2,4-butanediol) generally proved more effective in enhancing the rate of reaction than monofunctional alcohols such as methanol and *n*-propanol. However, not all alcohols are effective, *e.g.* benzyl alcohol (0.1 M) and the polyhydric alcohol inositol (0.4 M), had no effect on the rate. The most reactive alcohol among those studied, was pentaerythritol, which doubled the rate of the enzymatic reaction at a low concentration of 0.05 M. It was therefore chosen for further studies and compared with ethylene glycol and methanol.

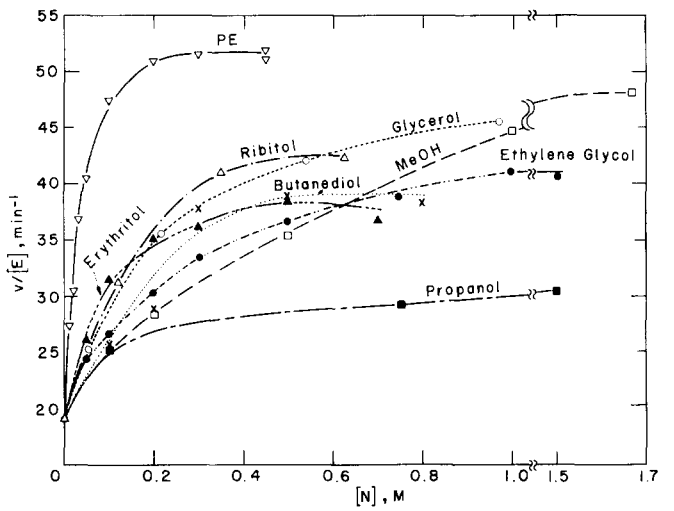


Fig. 1. The rate of release of phenol from 1.0 mM phenyl hippurate, catalyzed by chymotrypsin, in the presence of varying concentrations of alcohols, [N]. PE, pentaerythritol.

The chymotrypsin-catalyzed decomposition of phenyl hippurate in the presence of varying pentaerythritol concentrations was measured at three substrate concentrations (Fig. 2). The rates all increase and level off, but with higher substrate concentrations, higher plateaux are attained. Lineweaver–Burk plots of $1/v$ *vs* $1/$[phenyl hippurate] at each pentaerythritol concentration gave a series of parallel lines. From these plots the k_{cat} and K_m values were obtained. Although both k_{cat} and K_m values

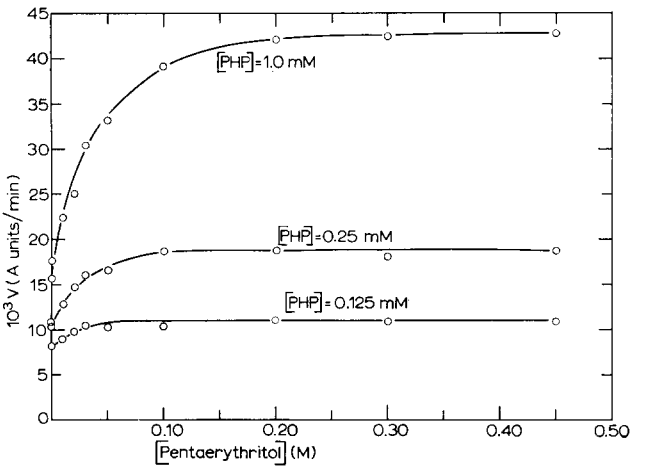


Fig. 2. The rate of release of phenol from phenyl hippurate (PHP), catalyzed by chymotrypsin, in the presence of varying concentrations of pentaerythritol, at different concentrations of phenyl hippurate.

TABLE I

THE KINETIC PARAMETERS FOR THE CHYMOTRYPSIN-CATALYZED DECOMPOSITION OF PHENYL HIPPURATE AT VARIOUS CONCENTRATIONS OF ADDED PENTAERYTHRITOL

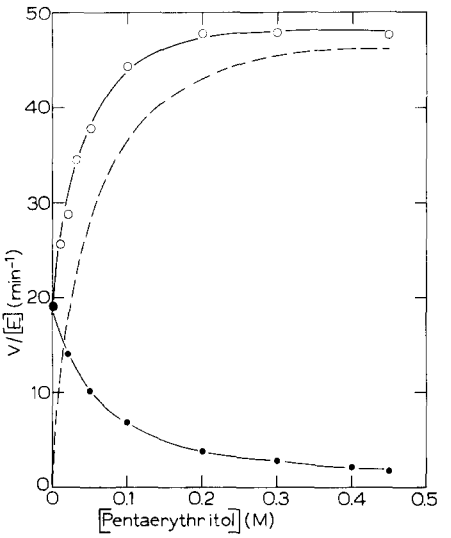
Concn of pentaerythritol (M)	$10^3 \cdot K_m$ *(M)*	k_{cat} *(min^{-1})*	$10^{-5} \cdot k_{cat}/K_m$ *($min^{-1} \cdot M^{-1}$)*
0	0.16	23	1.43
0.01	0.27	34	1.26
0.02	0.28	39	1.39
0.03	0.36	50	1.39
0.05	0.45	59	1.30
0.20	0.66	85	1.29
0.45	0.68	87	1.28

increase with added pentaerythritol, the ratio k_{cat}/K_m remains essentially constant (Table I).

Under the same experimental conditions the rate of release of protons was also measured at various pentaerythritol concentrations. It was found that this rate decreased sharply, *e.g.* at 0.4 M pentaerythritol, the rate was only 12% of that in the absence of alcohol (Fig. 3).

With ethylene glycol as a nucleophile, the same overall behavior was observed, *i.e.* increase in total rate and levelling off, while the rate of proton release decreased. However, with ethylene glycol the total rates also decrease slightly at higher concentrations of nucleophile. This decrease was more obvious as the substrate concentration was lowered, presumably due to competitive inhibition (see Discussion). In this case, the ratio k_{cat}/K_m was found to decrease with ethylene glycol concentration (Fig. 4).

On adding methanol, the total rate increased while that of hydrolysis decreased. In comparison with pentaerythritol and ethylene glycol, however, much



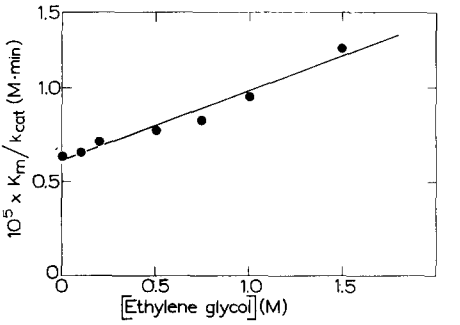


Fig. 3. The rate of release of phenol (○—○) and protons (●—●) from 1.0 mM phenyl hippurate in the presence of pentaerythritol, catalyzed by chymotrypsin. The dashed line is the difference of these rates and represents the rate of alcoholysis.

Fig. 4. The dependence of K_m/k_{cat} on the concentration of ethylene glycol, for the chymotrypsin-catalyzed hydrolysis of phenyl hippurate, determined at different concentrations of ethylene glycol.

higher concentrations of methanol were needed to affect both the rates of phenol and proton release.

DISCUSSION

Phenyl hippurate has been previously studied as a substrate for chymotrypsin. It was shown that the values of k_{cat} for 9 substituted phenyl hippurates (including phenyl hippurate) differed at most by approximately 10%[9]. In another study, 5 substituted phenyl hippurates (including phenyl hippurate) showed, within experimental error, the same values of k_{cat}[10]. This was proposed as strong evidence that in the rate determining step, there occurs the breakdown of a common intermediate, hippuryl-chymotrypsin.

In such cases when deacylation (k_3) is the slow step (Eqn 2), then added nucleophile will accelerate the release of phenol by nucleophilic attack on the intermediate acyl-enzyme, thus increasing its rate of breakdown. At the same time the rate of hydrolysis should not be affected[2–4]. Indeed this behavior was observed by Bender *et al.*[2] when adding methanol to *trans*-cinnamoyl-chymotrypsin.

However, in the present study of the effect of alcohols on the chymotrypsin-catalyzed hydrolysis of phenyl hippurate, a different behavior was observed. Although deacylation is rate determining for this substrate, and the total rate of reaction increases with added nucleophile, the rate of proton release decreases for the three alcohols studied. In addition, the total rates of reaction in the presence of added alcohols do not increase linearly, but tend to level off and reach a constant rate (Fig.

1). Thus, the simple Scheme II, as accepted for chymotrypsin[2], is insufficient for explaining the findings of this work, and must be modified.

The rate of phenol release in the presence of a nucleophile, v_{total}, is the sum of the rate of hydrolysis (v_H) and the rate of alcoholysis (v_N):

$$v_{total} = v_H + v_N \tag{3}$$

Taking, for example, the case of pentaerythritol as added nucleophile, the rates of phenol and proton release are shown in Fig. 3. The rate of alcoholysis was obtained by subtracting v_H from v_{total}. The shape of the curve for v_N is due to saturation of a postulated binding site for pentaerythritol on the enzyme. The binding constant (K_N) of pentaerythritol to this site was obtained from a double reciprocal plot (*i.e.* $1/v_N$ *versus* 1/[pentaerythritol]). The rate of hydrolysis in the presence of pentaerythritol showed a typical inhibition curve. The inhibition constant of hydrolysis, K_H, was obtained by plotting $1/v_H$ *versus* [pentaerythritol]. For comparison, the data for methanol and ethylene glycol were analyzed in a similar manner. The kinetic parameters obtained for these nucleophiles are shown in Table II.

TABLE II

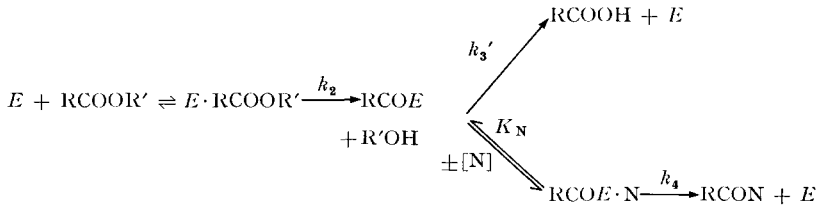
KINETIC PARAMETERS FOR CHYMOTRYPSIN-CATALYZED REACTIONS OF PHENYL HIPPURATE WITH ALCOHOLS

Phenyl hippurate concentration, 1.0 mM.

	Pentaerythritol	*Ethylene glycol*	*Methanol*
K_N (M)	0.054	0.34	0.94
K_H (M)	0.053	0.38	0.4
$k_4 = V_N/[E]$ (min^{-1})*	55	41	70
k_4/K_N ($M^{-1} \cdot min^{-1}$)	1020	120	74

* V_N is v_N extrapolated to infinite [N], obtained from a double-reciprocal plot.

The agreement, within experimental error, between the values of K_N and K_H for pentaerythritol and ethylene glycol suggests that the binding of the alcohol is responsible both for the alcoholysis and for the inhibition of hydrolysis. The following scheme is consistent with this hypothesis:



(4)

In this scheme the nucleophile binds to a site on the enzyme, thus inhibiting the reaction of water with the acyl–enzyme. Thus, the rate constant for alcoholysis, k_N, equals k_4 multiplied by the fraction of the acyl–enzyme which contains bound nucleophile:

$$k_N = \frac{v_N}{[E]} = k_4 \cdot \frac{[N]}{K_N + [N]} \quad (5)$$

Similarly, the hydrolysis rate constant, k_H, is related to the fraction of "free" acyl–enzyme:

$$k_H = \frac{v_H}{[E]} = k_3' \cdot \frac{K_N}{K_N + [N]} \quad (6)$$

since $K_H = K_N$.

These simple expressions can be shown to hold under two conditions: (i) $[S] \gg K_s$, (ii) $k_2 \gg (k_H + k_N)$. Since we worked at a phenyl hippurate concentration of 1 mM ($K_m = 0.16$ mM) condition (i) will hold, and as stated above, previous studies have indicated that deacylation is rate-limiting in this system.

An alternative explanation for the observed behavior in the presence of pentaerythritol would be a change in the rate-determining step. If the rates of acylation and deacylation were comparable and both steps contributed to the observed rate, then added nucleophile would enhance the rate of deacylation until acylation became rate-limiting. A levelling off of total rate corresponding to the acylation rate constant, k_2, would be observed. This same rate constant, k_2, would be reached regardless which nucleophile were reacting. (An example of such behavior was noted with papain (EC 3.4.4.10) and added nucleophiles)[11]. In the present study we can rule out this possibility, since various limiting rates are reached, depending on the nucleophile (Fig. 1).

Relative reactivites of alcohols

The bimolecular rate constant for attack of pentaerythritol and the other alcohols on the hippuryl–chymotrypsin intermediate can be derived from the initial slope of the curve for the total rate, or by dividing k_4/K_N for the alcoholysis (Table II). The rate constants for the attack of many oxygen and nitrogen nucleophiles on furoyl–chymotrypsin have been measured directly[5]. The nucleophiles studied included diols such as 1,4-butanediol and ethylene glycol but not pentaerythritol. The rate constants were all 1–2 orders of magnitude less than those determined in the present case. However, if a second-order rate constant is calculated for water attack by dividing k_3' by 55 M*, the ratios k_4/k_3 are of the same order of magnitude for the furoyl– and hippuryl–enzymes. It has recently been suggested[6] that the reactivity of nucleophiles compared to that of water is smaller for the more specific substrates. Indeed $k_{methanol}/k_{water}$ ratios for these two non-specific substrates are approximately 4-fold greater than those for specific ones[2].

The effect of alcohols on acylation and deacylation

The identical values of K_N and K_H (within experimental error), reflect their dependence on the same event, *i.e.* the simultaneous binding of nucleophile to the acyl–enzyme, and exclusion of water from its postulated site.

However, added nucleophiles can also compete with the substrate for the

* As pointed out[5] this has no meaning if water is binding and saturating a site.

active site on the free enzyme. Indeed it has been shown[12] that small organic molecules such as acetonitrile, acetone and dioxane can competitively inhibit chymotrypsin-catalyzed reactions. Similar effects were also observed in the present work with these molecules when using phenyl hippurate as substrate.

A useful criterion for discerning competitive inhibition is the ratio k_{cat}/K_m. When the nucleophile competes with the substrate for the enzyme, prior to acylation, the measured K_m will be increased by an additional factor of $(1 + [N]/K_i)$, where K_i is the inhibition constant of this nucleophile, whereas k_{cat} remains unchanged. This effect thus reflects events occurring prior to acylation. However, when a nucleophile attacks the acyl–enzyme, without causing any competitive inhibition, this ratio will remain constant and equal k_2/K_s, since the reaction of nucleophile will increase K_m and k_{cat} equally. This can be seen from the following equations which are analogous to those derived previously[3]:

$$k_{cat} = \frac{k_2 (k_N + k_H)}{k_2 + k_H + k_N} \tag{7}$$

$$K_m = \frac{K_s (k_N + k_H)}{k_2 + k_H + k_N} \tag{8}$$

For pentaerythritol, k_{cat}/K_m is independent of the nucleophile concentration (Table I), indicating the absence of competitive inhibition in the acylation process. Moreover, $K_N = K_H$ (Table II), indicating that the binding of pentaerythritol to the acyl–enzyme is equally responsible for inhibiting hydrolysis and promoting alcoholysis. For ethylene glycol, K_N is comparable to K_H (Table II), but in this case competitive inhibition occurs in the acylation step as reflected by the dependence of the ratio k_{cat}/K_m on nucleophile concentration (Fig. 4). A value of $K_i = 1.7$ M was obtained from this plot. This competitive inhibition for free enzyme will reduce the concentration of hippuryl–chymotrypsin, but will not alter its binding constant with the alcohol as expressed either by K_H or K_N. For methanol, the non-equivalence of K_N and K_H suggests that its binding has different effects on hydrolysis and on alcoholysis.

Nature of the nucleophilic site on chymotrypsin

In all previous work with nucleophiles added to chymotrypsin, the observed rates were linear with added nucleophile. It was, therefore, assumed that if a site for the nucleophile existed, it was not saturated[2,5]. We do not know of other cases where the ratio k_{cat}/K_m was examined in the presence of nucleophiles added to chymotrypsin. However, for papain, in cases where the ratio k_{cat}/K_m was found to increase, a prior binding of nucleophile (to free enzyme) was proposed[11,13].

Among the alcohols studied, pentaerythritol is the only effective nucleophile that does not show any trend for competitive inhibition with substrate. Two possible processes for the special action of this nucleophile, which can be envisioned, are: (1) on forming the acyl–enzyme the alcohol moiety of the substrate ester leaves, and is replaced by the nucleophile. (2) As the acyl–enzyme is formed, a new site for the nucleophile is generated. In both cases the nucleophilic site overlaps that from which water reacts, and therefore, on binding nucleophile, water can be excluded. Since pentaerythritol does not compete with the substrate for the active site on the free

enzyme, we favor the idea that a new site for the nucleophile is formed on the acyl–enzyme*. This would probably require a local conformational change.

It thus seems that the pentaerythritol molecule is endowed with a unique structure enabling it to serve as a probe for the nucleophilic site on acyl–chymotrypsins.

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* This hypothesis is supported by the fact that different values were obtained for binding of ethylene glycol to chymotrypsin (K_i = 1.7 M) and to hippuryl–chymotrypsin ($K_N = K_H$ = 0.36 ± 0.02 M).