

# Different kinetic patterns in the $\alpha$ -chymotrypsin-catalysed hydrolysis of synthetic ester substrates

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The reaction of  $\alpha$ -chymotrypsin with AcTyr-OEt and with AcTrp-OEt at pH 7.0 and 7.8 was studied over a wide range of substrate concentrations. The reaction with AcTyr-OEt at pH 7.8 was shown to be non-hyperbolic using a variety of criteria whereas those at pH 7.0 with the same substrate and at both pH values with AcTrp-OEt were hyperbolic. The non-hyperbolicity of the reaction with AcTyr-OEt at pH 7.8 followed a pattern of negative cooperativity with a Hill coefficient for the high substrate concentration range of 0.48. Although other explanations are possible, the pH dependence of the reaction with AcTyr-OEt could be related to the slow transition of the two known forms of the enzyme.

<i>Negative cooperativity</i>	<i>Nonhyperbolic kinetics</i>	<i><math>\alpha</math>-Chymotrypsin</i>	<i>Hyperbolic kinetics</i>	<i>Hill coefficient</i>
		<i>Slow transition</i>		

## 1. INTRODUCTION

Detailed study of the substrate dependence of enzyme activity has shown that many enzymes thought to obey hyperbolic kinetics follow a more complicated pattern. This is true not only of oligomeric enzymes but also of some monomeric enzymes such as bovine pancreatic ribonuclease A [1,2], carboxypeptidase A [3], carboxypeptidase B [4], trypsin [5] and  $\alpha$ -chymotrypsin [6]. Here, the non-hyperbolic behaviour of  $\alpha$ -chymotrypsin using AcTyr-OEt at pH 7.8 as substrate is reported. Under these conditions kinetic analysis shows the existence of negative cooperativity with this substrate. On the other hand, a hyperbolic behaviour was found with this substrate at pH 7.0 and also when AcTrp-OEt was used as substrate at both pH values.

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**Abbreviations:** AcTyr-OEt, *N*-acetyltyrosine ethyl ester; AcTrp-OEt, *N*-acetyltryptophan ethyl ester

## 2. MATERIALS AND METHODS

$\alpha$ -Chymotrypsin (EC 3.4.21.1) from bovine pancreas was from Sigma (St. Louis, MO) and was freed of low- $M_r$  contaminants by chromatography on Sephadex G-25 according to Yapel et al. [7]. AcTyr-OEt and *N*-*trans*-cinnamoylimidazole were also from Sigma. All other reagents were of analytical grade from Merck (Darmstadt).

The active enzyme concentration was determined by means of method A of Schonbaum et al. [8] using *N*-*trans*-cinnamoylimidazole.

All buffers were filtered through 0.2  $\mu$ m Sartorius membrane filters and degassed. The pH of the solutions was controlled before and after both treatments.

For the assays a stock solution of enzyme was prepared in 1 mM HCl and kept at 4°C. The enzyme concentration in the reaction mixture was in the range 25–45 nM in the experiments with AcTyr-OEt and 32 nM in the case of AcTrp-OEt. The hydrolysis of AcTyr-OEt was followed spectrophotometrically at 244 nm, instead of the usual

237 nm, because of the high absorbance at this wavelength.  $\epsilon_{237}$  and  $\epsilon_{244}$  were calculated in this work and found to be 327 and 120  $\text{M}^{-1} \cdot \text{cm}^{-1}$ , respectively. A value of  $\epsilon_{275} = 1317 \text{ M}^{-1} \cdot \text{cm}^{-1}$  for AcTyr-OEt [9] was used as reference for calculating the substrate concentration. Substrate concentrations were in the range 0.3–8.5 mM in 0.1 M Tris-HCl, pH 7.8, or in 0.1 M phosphate buffer, pH 7.0 or 7.8. A typical experiment was carried out as follows: 1 ml substrate was left in the thermostatted cell compartment at 25°C until no change in absorbance was noted. Then, 20  $\mu\text{l}$  of enzyme solution was added and the change in absorbance at 244 nm followed. Each concentration point was assayed in duplicate or triplicate. Due to the long duration of the whole procedure the stock solution of enzyme was titrated 3 times at regular intervals (every 3–4 h) and, at the same time, an enzymic assay at an intermediate concentration of substrate was also carried out. No significant variations were found in either case. The progress curves were linear for at least 2–3 min.

In the case of AcTrp-OEt the reaction was followed with a Perkin-Elmer 650 spectrofluorimeter interfaced to a Perkin-Elmer Data Station 3600. The reaction conditions used were 0.1 M phosphate buffer, pH 7.0 or 7.8, at 25°C. The enzyme concentration in the assay mixture was 32 nM and the substrate concentrations were in the range 0.01–3.3 mM as measured at 300 nm using  $\epsilon_{300} = 693 \text{ M}^{-1} \cdot \text{cm}^{-1}$  [10]. In a typical assay, to 3 ml substrate solution equilibrated inside the thermostatted cell compartment for 5 min, 0.1 ml enzyme solution was added. Excitation was set at 315 nm (slit 6 nm) and emission at 366 nm (slit 4 nm) which are the corresponding maxima in the difference spectra between product and substrate. To compensate for possible drift, high sensitivity and a mode ratio were used and to avoid inner filter effects the absorbance of the substrate was never higher than 0.05.

### 3. RESULTS AND DISCUSSION

The concentration dependence of the  $\alpha$ -chymotrypsin-catalysed hydrolysis of AcTyr-OEt at pH 7.8 is shown in figs 1 and 2 using Eadie-Hofstee and Hanes plots, respectively. One can see clear deviation from linearity. Data processing by means of non-linear regression, using the

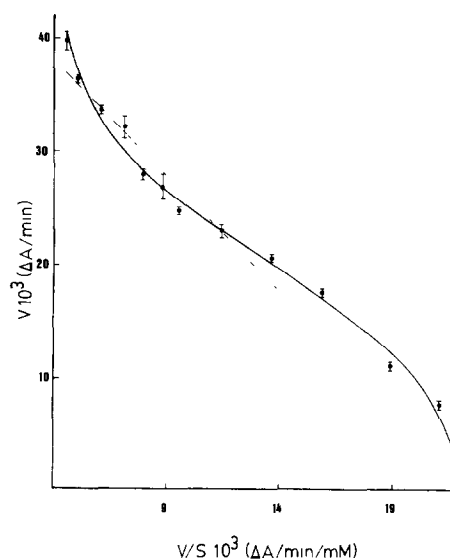


Fig.1. Substrate concentration dependence of the hydrolysis of AcTyr-OEt in 0.1 M Tris-HCl, pH 7.8. (●) Observed values; (—) predicted from a 2:2 rational function without weighting. (---) Result of adjusting the experimental data to the linearized Michaelis-Menten equation.

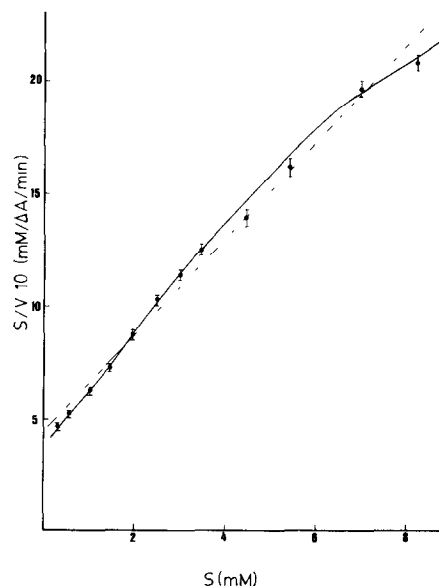


Fig.2. Hanes plot of the same data. Symbols as in fig.1.

BMDP3R programme [11], shows a very good fit with a rational function of a degree 2:2 [12]. The deviations from linearity can in both cases be interpreted as strong evidence for the existence of negative cooperativity. Typical hyperbolic behaviour with a  $K_m = 2.17$  mM was found with this substrate at pH 7.0.

Several tests were carried out to ensure the non-artefactual nature of the non-hyperbolic behaviour found at pH 7.8: (i) the profile is reproducible and the experimental error low as shown by the error bars (fig.1); (ii) the enzyme was subjected to gel filtration to remove any inhibitory material derived from autolysis; (iii) strict control of the enzyme activity was made throughout the experiment (see section 2); (iv) the controls recommended [13] in the case of 'downwards' curvatures in the double-reciprocal plots were carried out, namely, (a) the activity as a function of enzyme concentration in the range 26–225 nM is linear and goes through the origin, which means that it is not the end of an exponential, thus demonstrating that the enzyme solution is not contaminated by the substrate; (b) strict controls showed that there was no spontaneous hydrolysis; (c) in the range of substrate concentration used the Lambert-Beer law was always obeyed; (v) by working at 244 nm it was possible to carry out all measurements in 1 cm light-path cuvettes, thus avoiding some diffusion, mixing and adsorption problems that sometimes appear when using the narrower 2 mm light-path cuvettes. The possibility that the non-hyperbolic profile was due to the presence of enzyme dimers and/or oligomers is not likely as the concentration of dimers in dilute solutions is negligible [14]. In addition, similar results were obtained with AcTyr-OEt at pH 7.8 with both Tris-HCl or phosphate buffers although the rate was higher in the latter.

As already stated the shapes of the different graphs point to the existence of negative cooperativity in the  $\alpha$ -chymotrypsin-catalysed hydrolysis of AcTyr-OEt at pH 7.8. To confirm this, the Hill coefficient was determined using the method of Endrenyi et al. [15]. This method has the advantage that there is no need for an accurate value of the maximum velocity which is always difficult to obtain when there is negative cooperativity. Two intersecting straight lines were found, meaning that the Hill equation is not strictly followed in the whole range although the method

is applicable to each line separately [15]. In this way Hill coefficients of 1.12 and 0.48 for the low and high concentration range, respectively, were found thus confirming the existence of negative cooperativity. In the case of the hydrolysis of AcTrp-OEt clear hyperbolic behaviour was found at both pH 7.0 and 7.8 with a  $K_m$  value of 107 and 115  $\mu$ M, respectively, which are very close to those found by Zerner et al. [10].

$\alpha$ -Chymotrypsin has usually been considered a classical Michaelian enzyme and the values for the kinetic parameters  $k_{cat}$  and  $K_m$ , as well as other individual rate constants, have been determined on this assumption [10,16–18]. Substrate activation of  $\alpha$ -chymotrypsin was reported by Arihood and Trowbridge [19] using *p*-toluenesulphonyl-L-arginine methyl ester, a trypsin substrate and allosteric activation in the  $\alpha$ -chymotrypsin-catalysed hydrolysis of amide substrates was found with a variety of synthetic azobenzene compounds having bis-quaternary nitrogens [20]. No allosteric activation of ester substrates was found. Negative cooperativity has only been found with  $\delta$ -chymotrypsin [6] using *N*-carbobenzoxy-L-tryptophan *p*-nitrophenyl ester at pH 9.00 and 25°C. These authors only found negative cooperativity with the substrate containing tryptophan but not with its tyrosine-containing analogue and with the former the phenomenon was even restricted to a narrow range of temperature and ionic strength.

As in the case of  $\delta$ -chymotrypsin, negative cooperativity could be explained in terms of a slow transition mechanism such as that proposed by Ainslie and Neet [6] in which there are 2 forms of the enzyme corresponding to the neutral and high pH forms of the enzyme found by Fersht and Requena [21]. Based on the values given for the  $pK$  of the transition between the 2 forms (8.76) and the proportion of active and inactive forms, the proportion of inactive form at pH 7.8 must be approx. 25% [21] which is a rather significant amount. The proportion at pH 7.0 would be 10%. This, together with the slow rate constant for the isomerization ( $1.9$  s<sup>-1</sup> at pH 7.84 and  $3.1$  s<sup>-1</sup> at pH 6.84) [21] as compared to the deacylation rate constant ( $200$  s<sup>-1</sup> and  $120$  s<sup>-1</sup> at pH 7.8 and 7.0, respectively) [16,17] can explain the negative cooperativity found. Obviously, other explanations such as the allosteric mechanism described

for ribonuclease A [2] or the binding of substrate to subsites  $S_1$ ,  $S_2$ , etc. are also possible. However, in these cases it would be more difficult to explain the observed pH dependence.

The results found with AcTyr-OEt at pH 7.0 and with AcTrp-OEt at both pH values could be explained as being due to other factors, such as the different ratios between the values of the individual rate constants, that can mask a cooperative effect [6]. It is also interesting to note that Ainslie and Neet [6] found negative cooperativity with the substrate containing tryptophan and not with that containing tyrosine which is just the reverse of what has been found here. This should not be too surprising in view of the fact that the observation of negative cooperativity is restricted to narrow ranges of pH, temperature and ionic strength [6]. Experiments are in progress to gather more data on the non-hyperbolic behaviour of  $\alpha$ -chymotrypsin and thus be able to discriminate between different possible mechanisms.

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