

The Combined Effect of Thiocyanate Ions and Rise in Temperature on the Action of α -Chymotrypsin on Tyrosine Ethyl Ester

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The splitting of tyrosine ethyl ester by α -chymotrypsin can be described fairly well by two kinetic constants representing a zero'th and a first order term if followed to 95 % completion. Changing the ionic strength with KCl, NaCl, and K_2SO_4 leads to a decrease in the first order rate constant, both at 25°C and 38°C. Changing the concentration of KSCN gives a pattern resembling that of the other ions at 25°C but at 38°C the reaction follows a different pattern, giving an increase in the first order rate constant and a decrease in the zero'th order rate constant.

In a previous publication on the action of α -chymotrypsin on tyrosine ethyl ester Graae and Hansen¹ have discussed the deviations from the simple Michaelis-Menten reaction scheme. In order to investigate further this reaction the influence of ionic strength and temperature has been studied. Both the tryptic and the chymotryptic activity have been studied by several authors as a function of these two variables, but only by the initial rate method.²⁻⁴ We have therefore found it reasonable to reinvestigate these reactions by analysing the entire course of the reaction. Our results indicate that misleading conclusions have often been drawn when the effect of added ions on the rate of an enzymatic reaction is described in terms of "per cent inhibition".

EXPERIMENTAL

Crystalline α -chymotrypsin was kindly placed at our disposal by NOVO Terapeutisk Laboratorium, Copenhagen, Denmark. L-Tyrosin ethyl ester, HCl (TEE) were commercial preparations from Nutritional Biochemical Corporation, U.S.A. and Sigma, U.S.A. We

found no observable differences between the two preparations, neither when used as bought nor after recrystallisation. The experiments were carried out in 30 ml of the salt solution in which the desired amount of substrate was dissolved. By means of KOH pH was adjusted to 6.83, the pH optimum in 0.1 M KCl at 27°C.¹ The reaction was initiated by the addition of the enzyme solution and followed by continuous automatic titration in a self-recording pH stat. (Titration outfit consisted of PHM 26 and TTT 11 from Radiometer, Denmark, equipped with a Wetzer digital recorder, and 1 ml Summit micro-syringe). The titrations were performed with approximately 1 M KOH with the reaction chamber submerged in a water thermostat. The degree of reaction, α , is calculated as the proportion between the titration value at a given time, t , and the titration value after complete hydrolysis.

RESULTS

If the reaction follows a Michaelis-Menten scheme, or any other reaction scheme that gives rise to only a zero and a first order term, then the chronometric integral can be written

$$E \cdot t = A \cdot \alpha - B \ln(1 - \alpha)$$

where E = total enzyme concentration. The constants A and B are determined from plots of t/α versus $(\ln(1 - \alpha))/\alpha$.

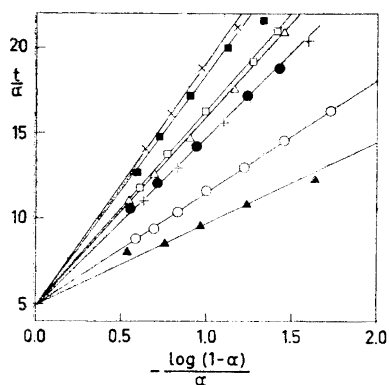


Fig. 1. t/α versus $-(\log(1 - \alpha))/\alpha$ for experiments in KCl. All experiments 40 mg TEE. 50 μ l chymotrypsin solution prepared by dissolving 30 mg α -chymotrypsin in 5 ml (10^{-4} M HCl + 0.2 M KCl). KCl concentrations: \blacktriangle 2.0 M; \circ 1.0 M; \bullet 0.2 M; $+$ 0.2 M; \triangle 0.1 M; \square 0.05 M; \blacksquare 0.02 M; \times 0.0063 M. Temperature 38°C.

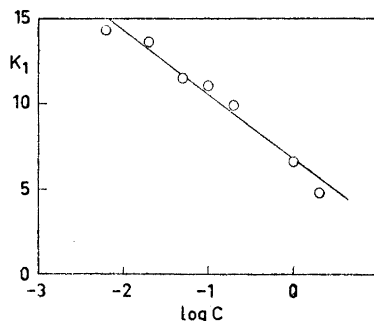


Fig. 2. Slopes from Fig. 1 versus $\log [KCl]$.

The effect of varying the concentration of KCl at 38°C is seen from Fig. 1. Disregarding minor deviations from the straight line it is seen that the zero order constant is independent of the salt concentration but that the first order constant decreases with increasing salt concentration. A plot of the first order constant versus the logarithm of the salt concentration shows a straight line, see Fig. 2. The effect seems to follow the ionic strength and to

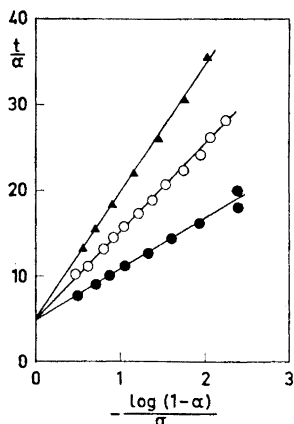


Fig. 3. Experiments similar to those recorded in Fig. 1, but with NaCl instead of KCl. NaCl concentrations 1.0, 0.1 and 0.01, respectively.

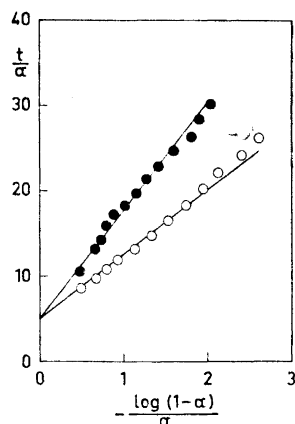


Fig. 4. Experiments similar to those recorded in Fig. 1, but with K_2SO_4 instead of KCl. K_2SO_4 concentrations 0.333 and 0.0033, respectively.

be independent of ionic individualities as indicated by Figs. 3 and 4 which show similar experiments in NaCl and K_2SO_4 .

In KSCN at 38° , the pattern is quite different, see Fig. 5. In 1 M KSCN the zero order coefficient is negative and the first order coefficient very large.

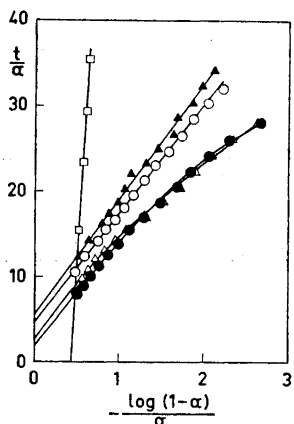


Fig. 5. Experiments similar to those recorded in Fig. 1, but with KSCN instead of KCl. \square 1.0 M; \triangle 0.3 M; \bullet 0.1 M; \circ 0.03 M; \blacktriangle 0.01 M. Enzyme solution prepared without KCl. Temperature $38^\circ C$.

The results obtained in KSCN at 25° , however, are similar to those in KCL at 25° and in KCl at 38° .

Two series of experiments with varying initial substrate concentration were therefore performed in 1 M KSCN at 25° (Fig. 6) and at 38° (Fig. 7). The

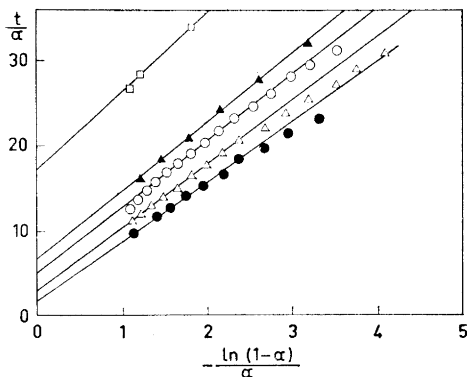


Fig. 6. The effect of variation of TEE concentration at 25°C in 1 M KSCN. TEE concentrations ● 10 mg/30 ml; Δ 20 mg/30 ml; ○ 30 mg/30 ml; \blacktriangle 40 mg/30 ml; \square 100 mg/30 ml. 50 μ l α -chymotrypsin solution prepared by dissolving 18 mg α -chymotrypsin in 3 ml 10^{-4} M HCl.

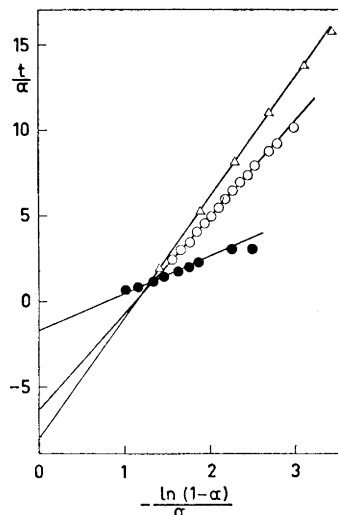


Fig. 7. The effect of variation of TEE concentration at 38°C in 1 M KSCN. TEE concentrations ● 10 mg/30 ml; ○ 30 mg/30 ml; Δ 40 mg/30 ml. Enzyme concentration the same as in the experiment recorded in Fig. 6.

zero and first order constants calculated from these two series are plotted versus the initial substrate concentration on Figs. 8 and 9.

Both Figs. 8 and 9 show, as described earlier,¹ that the zero order coefficient is proportional to and the first order constant linearly dependent on the

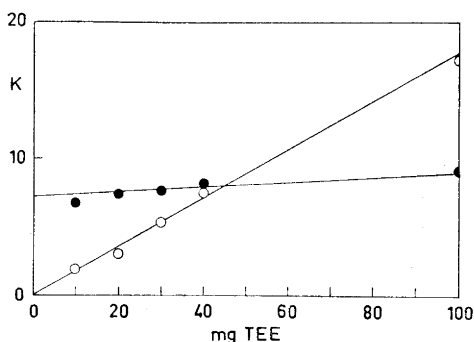


Fig. 8. Intercepts ○ and slopes ● from Fig. 6 versus initial substrate concentrations.

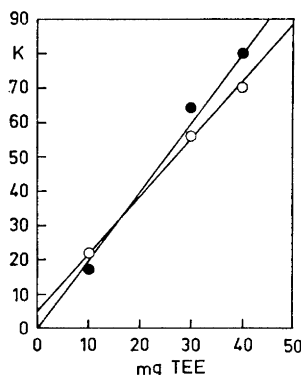


Fig. 9. Intercepts ● and slopes ○ from Fig. 7 versus initial substrate concentrations.

initial substrate concentration. It is seen that the ratio between the slope and the intercept of the line representing the first order constant is changed from a value near zero at 25° (Fig. 8) to a value about 2 (Fig. 9) at 38°.

DISCUSSION



By simple mathematical treatment the Michaelis-Menten scheme gives rise to the following chronometric integral where time, t , is a function of the degree of reaction, α :

$$E \cdot t = \frac{1}{k_2} a\alpha + \left(\frac{1}{k_1} + \frac{k_{-1}}{k_1 k_2} \right) \ln(1-\alpha) \quad (\text{a})$$

a is the initial substrate concentration and k_n is the velocity constant for the n 'th reaction.

As the k 's are positive it is obvious that this scheme cannot explain the occurrence of negative zero order constants, and an extension of the scheme is therefore required as for instance:



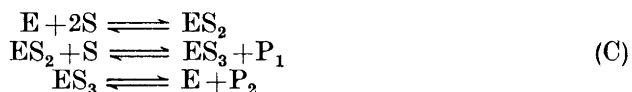
where the reaction products are split off consecutively. The corresponding chronometric integral is:

$$E \cdot t = \left(\frac{1}{k_2} - \frac{k_{-1}}{k_1 k_2} \right) a\alpha - \left(\frac{1}{k_1} + \frac{k_{-1}}{k_1 k_2} a \right) \ln(1-\alpha) \quad (\text{b})$$

For the reaction in question, a more complicated function has previously been suggested¹ in order to explain the deviations from the zero/first order form:

$$\begin{aligned} E \cdot t &= \left(\frac{1}{k_3} - \frac{k_{-2}}{k_2 k_3} \right) a\alpha - \left(\frac{1}{k_2} + \frac{k_{-2}}{k_2 k_3} a \right) \ln(1-\alpha) + \left(\frac{1}{k_1} - \frac{k_{-1} k_{-2}}{k_1 k_2 k_3} \right) \frac{1}{a} \left(\frac{1}{1-\alpha} - 1 \right) \\ &+ \frac{1}{2} \left(\frac{k_{-1}}{k_1 k_2} + \frac{k_{-1} k_{-2}}{k_1 k_2 k_3} a \right) \frac{1}{a^2} \left(\frac{1}{(1-\alpha)^2} - 1 \right) \end{aligned} \quad (\text{c})$$

This function corresponds to the following three consecutive reactions:



The effect demonstrated in Figs. 1–4 can be explained according to scheme B by assuming an increase in k_1 and k_{-1} as compared with k_2 and by scheme (C) as an increase in k_2 and k_{-2} as compared with k_3 . In both cases this corresponds to a relative stabilization of the complexes ES_3 and ES , respectively. Trypsin shows in certain cases a similar behaviour.⁵ The specific action of SCN^- ions can be described according to scheme B as an increase of k_{-1} as

compared to k_1k_2 and in reaction scheme (C) in k_{-2} as compared to k_2k_3 . In both cases this means that SCN^- ions favour the decomposition of the active complex to give substrate and enzyme instead of products and enzyme. It is seen from the suggested reaction scheme that the increase in the mentioned velocity constants will give rise to both a negative zero order term and a more marked dependence of the first order constant on initial substrate concentration.

It is not possible to explain the effect by assuming an irreversible inhibition of the enzyme. If such an inhibition took place immediately after the enzyme was placed in the KSCN solution it is seen from eqns. (a), (b), and (c) that the ratios between the different coefficients should be constant, but the coefficients themselves enlarged. That this is not the fact is seen from Figs. 3 and 5. If such an inhibitory process did not take place immediately, the same figures indicate that the ratios between the coefficients would be different at different degrees of reaction, and straight lines would not be produced in the used plots.

In Fig. 5 obvious deviations from the straight line are seen during the entire course of the reaction at 0.1 M and 0.3 M KSCN. It is, however, not likely that slow irreversible inactivation should be the explanation in these cases, as the "thiocyanate effect" is produced both at lower concentrations (0.01 M and 0.03 M) and at higher concentrations (1.0 M) without notable deviation from the straight line.

It is more likely that the deviations are caused by the fact the eqns. (a) and (b) only represent a part of the integrated rate equation, so that the deviations from (b) at certain SCN^- concentrations become more apparent. (Observe that Figs. 1–4 are calculated with \log_{10} and Figs. 5–7 with \ln). It should be mentioned that it has no meaning to describe the inhibitory effect of SCN^- ions in terms of "percent inhibition". If, for example, an experiment has an initial velocity of 50 % of the initial velocity found in KCl medium, the experiment will by no means continue in the same way as an experiment in KCl with half enzyme concentration.

To find out whether the action could be attributed specifically to the enzyme or to the substrate, UV spectra were recorded for both enzyme and substrate in 1 M KCl and 1 M KSCN. The enzyme showed a maximum at 281 nm in both solutions and the substrate similarly at 275 nm but the descent of the curves towards shorter wavelength were for both compounds strongly influenced by the ionic media, being much steeper in KSCN than in KCl. Exactly the same effect was found in solutions of phenol (maximum absorbance at 270 nm). From spectral evidence, therefore, the influence of SCN^- ions may be either through interaction with the enzyme or with the substrate or both.

The possibility that the effect is due to the existence of a free α -aminogroup in the substrate has been investigated by using *N*-acetyl tyrosine ethyl ester as substrate. SCN^- ions have a pronounced effect also with this substrate, but the kinetics turns out to be so much different that these investigations will be the subject of a separate publication.

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