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Ficin-catalysed Reactions: the Affinity of Ficin for some Arginine Derivatives

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We have recently obtained detailed information about the kinetics and mechanism of the trypsin-catalysed hydrolysis of α-benzoyl-L-arginine ethyl ester and amide to α-benzoyl-L-arginine (Gutfreund, 1955*a*; Bernhard, 1955*a*, *b*). It appeared likely that further insight into the mechanism of enzyme-catalysed hydrolysis reactions could be gained from a comparison of the reactions of two enzymes which are specific for the same substrates. During the course of their pioneer work on the specificity of peptidases Irwing, Fruton & Bergmann (1941) have shown that ficin, an enzyme isolated from fig-tree latex, has the same specificity as trypsin and catalyses the hydrolysis of benzoyl-L-arginine amide. The conditions required for optimum activity of ficin were so different from those of trypsin that one could conclude that the two enzymes catalyse the same reactions via different mechanisms.

Results obtained from varied studies of ficin-catalysed reactions will allow us to map out the steps involved in the formation of the enzyme–substrate complex and its decomposition to enzyme and products. In the present paper we describe the effect of pH on the catalytic activity and on the affinity of ficin for benzoyl-L-arginine ethyl ester, benzoyl-L-arginine amide and benzoyl-L-arginine.

EXPERIMENTAL

Substrates

Benzoyl-L-arginine (BA), its amide (BAA) and its ethyl ester (BAEE) were prepared by the method of Bergmann, Fruton & Pollok (1939).

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The concentration of a nearly saturated stock solution of the zwitterion BA was determined as follows. A sample of BA was dissolved in water and titrated to pH 7·0 with 0·1 N-NaOH. The mixture was allowed to stand at 5° for 2 days and then filtered; the filtrate was brought back to pH 2·0 by the addition of N-HCl and the solution was titrated potentiometrically with 0·1 N-NaOH. The BA in this solution was found to be 0·02 M and its pK_A was $3{\cdot}40 \pm 0{\cdot}05$ at 25°.

Ficin

Crude ficin (100 g.) (L. Light and Co. Ltd., Colnbrook, Bucks) is suspended in 1 l. of 0·01 N-HCl and stirred occasionally for 24 hr. The suspension is then dialysed against running tap water for 24 hr. and filtered. To 100 ml. of the filtrate is added 30 g. of $(NH_4)_2SO_4$ and the mixture is left to stand at +4° for 2 hr. and then filtered. The precipitate is washed with a solution containing 24·5 g. of $(NH_4)_2SO_4$/100 ml., and redissolved in 400 ml. of distilled water, dialysed against distilled water until salt-free and finally dialysed against a mixture of 0·01 N-HCl and 0·1 M-NaCl. This enzyme stock solution was found to be of constant activity when stored at 4° for 2 months.

A 1% solution of the enzyme in 0·1 M sodium phosphate buffer, pH 6·7, was examined in the ultracentrifuge; this was kindly done for us by Mr Per Bro of Yale University. The protein sedimented as one homogeneous boundary with a sedimentation constant S_{20} of $2{\cdot}56 \times 10^{-13}$, corrected to sedimentation in water at 20°. Preliminary osmotic pressure measurements of ficin solutions in 0·1 M sodium phosphate, pH 5·05, indicate a mol.wt. of approx. 26 000. On the basis of this mol.wt. the enzymic activity/mole of protein was calculated for

the hydrolysis of BAEE under optimum conditions of pH, substrate concentration and activation with cysteine and ethylenediaminetetraacetic acid (EDTA) (see Results). It was found to be 1·4 moles of BAEE hydrolysed/sec./mole^{-1} of enzyme.

Kinetic measurements

The course of the hydrolysis of BAEE was followed by potentiometric titration. The details of the method used were recently described by one of us (Bernhard, 1955*a*). It was found that both cysteine and versene had to be added to the reaction mixture in order to obtain maximum activity. In this respect the ficin system resembled papain. Dr E. L. Smith kindly communicated to us his experiences with the activation of papain some time before the publication of the paper by Kimmel & Smith (1954). All experiments described here were carried out at 25° in 20 ml. of solution, 5×10^{-3}M in cysteine, 1×10^{-3}M in EDTA and 0·15M in NaCl. The buffering capacity was adequate over the pH range 3·5–8·0. Increasing or decreasing the concentration of either EDTA or cysteine (or of both) by a factor of 10 did not affect the rate of the ficin-catalysed reactions.

RESULTS

The stability of the enzyme, the favourable pH range and relatively high values of the Michaelis constant, K_m, and the inhibition constants, K_I, have resulted in unusually good kinetic data.

Some typical results of rate measurements of ficin-catalysed hydrolysis of BAEE under specified conditions of pH and initial substrate concentration $[S]_0$ are shown in Fig. 1.

The Michaelis constant for the ficin–BAEE system was determined at various pH values by the method of Lineweaver & Burk (1934). For the calculation of K_m and $V_{max.}$ (the limiting value at high substrate concentration) from their equation

$$\frac{1}{V_0} = \frac{1}{V_{max.}}\left(\frac{K_m}{[S]_0} + \frac{1}{V_{max.}}\right), \qquad (1)$$

data for V_0 (the initial velocity) have to be obtained over a wide range of $[S]_0$. Plots of $1/V_0$ against $1/[S]_0$ for measurements between pH 3·80 and 6·50 are shown in Fig. 2. The values calculated from these plots show that $K_m = 2{\cdot}3 \times 10^{-2}$M and is invariant over this pH range, while there is a nearly sevenfold change in $V_{max.}$ (Fig. 3). In the above determinations of K_m competitive inhibition

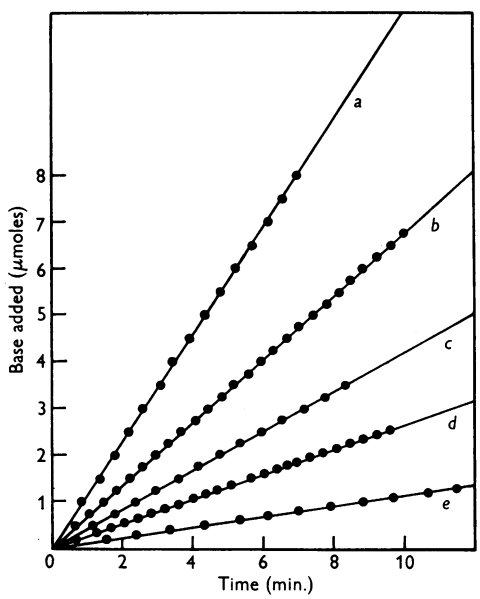

Fig. 1. Ficin-catalysed hydrolysis of benzoyl-L-arginine ethyl ester (BAEE) in 5×10^{-3}M cysteine, 10^{-3}M EDTA and 0·15M-NaCl. *a*, 0·025M BAEE, pH 6·5; *b*, 0·025M BAEE, pH 4·5; *c*, 0·006M BAEE, pH 5·5; *d*, 0·0029M BAEE, pH 6·5; *e*, 0·0029M BAEE, 0·061M BAA, pH 5·5.

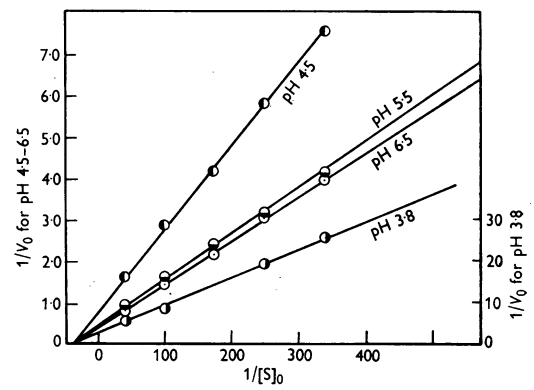

Fig. 2. Plots of the reciprocal of the initial rate, $1/V_0$, versus the reciprocal of the initial substrate concentration, $1/[S]_0$, at 25·0° in 5×10^{-3}M cysteine, 10^{-3}M EDTA, and 0·15M NaCl at various pH values.

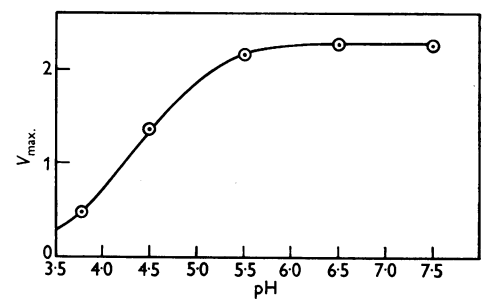

Fig. 3. Plot of the limiting rate, $V_{max.}$ of BAEE hydrolysis as a function of pH. The solid line is the theroretical ionization curve of a group with $pK = 4{\cdot}35$.

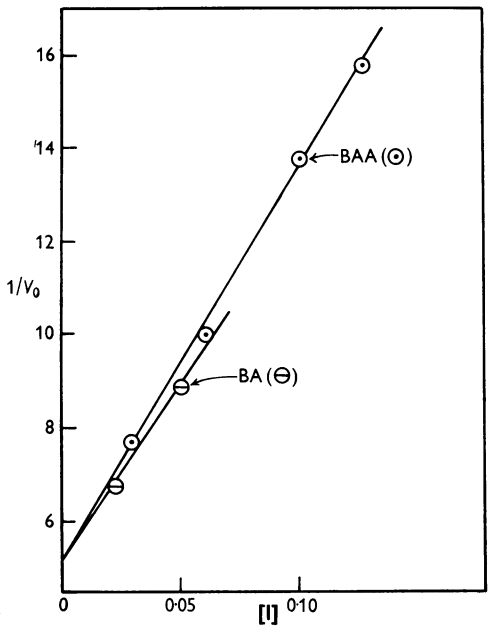

Fig. 4. Reciprocal of the initial rate, $1/V_0$, of hydrolysis of 3×10^{-3} M BAEE as a function of inhibitor concentration. ⊖, Benzoyl-L-arginine (BA); ⊙, benzoyl-L-arginine amide (BAA).

by products was not considered, since the limit of product concentration was never more than a few per cent of $[S]_0$.

The inhibition of the hydrolysis of BAEE by BAA and BA was studied at pH 5·5. The plots of $1/V_0$ against [I] (inhibitor concentration) at constant concentration of BAEE ($[S]_0 = 3 \times 10^{-3}$ M) are shown in Fig. 4. The inhibition by the molecular acid BA could not be determined by our method, which becomes insensitive in the presence of appreciable concentrations of buffering substances. At pH values where BA is present in significant concentration with its carboxyl group un-ionized, its buffering power would be excessive.

K_I was calculated from the equation (Lineweaver & Burk, 1934):

$$\frac{1}{V_0} = \frac{1}{V_{max.}}\left(\frac{K_m}{[S]_0} + 1 + \frac{K_m[I]}{K_I[S]_0}\right). \quad (2)$$

The results obtained were:

for BAA, $K_m/K_I = 0{\cdot}43$, $K_I = 5{\cdot}4 \times 10^{-2}$ M;
for BA, $K_m/K_I = 0{\cdot}38$, $K_I = 6{\cdot}0 \times 10^{-2}$ M.

DISCUSSION

From the classical Michaelis–Menten scheme

$$E + S \rightleftharpoons ES \rightarrow E + P, \quad (3)$$

the expression for the rate of appearance of product $d[P]/dt$ at a given total enzyme concentration $[E]_0$ is

$$d[P]/dt = k_r[E]_0[S]/(K_m + [S]). \quad (4)$$

Usually some subscript number is assigned to the rate constant k; however, it has been pointed out recently by Smith, Finkle & Stockell (1955) and Gutfreund (1955*a*, *b*) that the formation and decomposition of the enzyme–substrate complex ES is better described by a number of steps. The constant k_r in equation (4) refers to the rate-determining step of the series and under different conditions a different step may be rate determining.

A reduced SH group is necessary for the catalytic activity of ficin; from preliminary experiments (Hammon & Gutfreund, unpublished work) it has been concluded that the enzyme is inactivated mole/mole by methyl mercury. The remarkable efficiency of ficin as a catalyst for transfer reactions and the fact that this enzyme catalyses the hydrolysis of BAEE and BAA at approximately the same rate (Forrest, Sturtevant & Gutfreund, unpublished observations) leads one to set up a scheme for the hydrolysis mechanism which can be used as a working hypothesis for the planning of further experiments:

$$E + S \underset{k_1}{\overset{k_1}{\rightleftharpoons}} (ES)_1 \xrightarrow{k_2} (ES)_2 \xrightarrow{k_3} E + P_2 \quad (5)$$
$$\qquad\qquad\qquad\qquad\qquad\quad +$$
$$\qquad\qquad\qquad\qquad\qquad\quad P_1$$

$(ES)_1$ is a loose complex formed by the initial adsorption of the substrate on the specificity site of the enzymes. The second-order rate constant ($k_1 = 5 \times 10^2$ l./mole/sec.) of the formation of $(ES)_1$ has recently been determined by Gutfreund (1955*b*) from studies of the pre-steady state kinetics. The formation of $(ES)_2$ is assumed to involve a thiol-ester bond between the SH group of the enzyme and the acyl group of the substrate and the concomitant liberation of P_1, which would be EtOH or NH_3 in BAEE and BAA respectively. The fact that such an enzyme thiol ester would be more stable than the iminazole-acyl compound, which was proposed as an intermediate in trypsin reactions (Gutfreund, 1955*a*) would explain the following differences in the kinetic behaviour of trypsin and ficin-catalysed reactions. First the longer half-life of acylated ficin is more suitable for a transfer reaction, and secondly its slow rate of hydrolysis makes this the rate-determining step which would be equivalent in the ester and amide hydrolysis. It has been shown that in the reactions of trypsin the catalytic attack of the active group of the enzyme on the carbonyl carbon of the substrate is likely to be the rate-determining step. The mechanism suggested for the ficin-catalysed reactions would require k_2 to be very much faster in

ester hydrolysis than in amide hydrolysis. Preliminary studies by the methods suggested by one of us (Gutfreund, 1955*b*) indicate that this is the case and a detailed investigation of the rate of formation of $(ES)_2$ by ficin with ester and amide substrates is in progress.

When k_3 is the rate-determining velocity constant the Michaelis constant is determined by the steady-state concentration of $(ES)_2$. For the ficin-catalysed hydrolysis of BAEE, $k_3[E]_0$ changes with pH over a range in which K_m is very accurately invariant. This shows that the inhibition by H^+ ions is truly non-competitive and that the formation of $(ES)_2$ is not affected by pH. The rate of decomposition of $(ES)_2$ to $E+P_2$ is, however, pH-dependent.

Fig. 3 shows the relation between $V_{max.}$ and pH, indicating half optimum activity at pH 4·35. The solid line is a calculated ionization curve for a group of $pK = 4{\cdot}35$. It appears, therefore, that an ionized carboxyl group, probably the free carboxyl of glutamic or aspartic acid, plays a dominant role in the rate-determining hydrolysis of the acyl-thiol enzyme–substrate compound. Since $V_{max.}$ is constant over the range of pH 6–7·5 one can conclude that neither ^+H_3O nor OH^- ions are involved in the rate-determining hydrolysis of $(ES)_2$.

It is evident that ficin catalyses the hydrolysis of BAEE and BAA by a widely different mechanism from that of trypsin. The binding constants for BAEE, BAA and BA on ficin are remarkably similar. A detailed discussion of the causes of this effect will be given when the rate constants for the three steps for both BAA and BAEE hydrolysis have been obtained from pre-steady state studies.

SUMMARY

1. The kinetics of the ficin-catalysed hydrolysis of benzoyl-L-arginine ethyl ester have been studied under varied conditions.

2. From effects of pH on $k_r[E]_0$ and K_m it has been concluded that hydrogen ions act as non-competitive inhibitors on this enzyme, and that an ionizing group with $pK = 4{\cdot}35$ plays a dominant role in the rate-determining step of the catalysis mechanism. $K_m = 2{\cdot}3 \times 10^{-2}$, and is constant over the range of pH 3·8–6·5.

3. All the available evidence of the reactions of ficin has been used to set up a scheme for the path of the reaction between ficin and its substrates.

4. The affinities of ficin for benzoyl-L-arginine and its ester and amide have been compared by the determination of the Michaelis constant for the ester hydrolysis ($K_m = 2{\cdot}3 \times 10^{-2}$) and the inhibition of the ester hydrolysis by benzoyl-L-arginine ($K_I = 6{\cdot}0 \times 10^{-2}$) and benzoyl-L-arginine amide ($K_I = 5{\cdot}4 \times 10^{-2}$).

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Observations on the Occurrence of 16-*epi*oestriol in Urine

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16-*epi*Oestriol (oestra-1:3:5-triene-3:16β:17β-triol) was recently isolated from the urine of pregnant women by Marrian & Bauld (1954, 1955). However, the yield was small and the isolation procedure was somewhat rigorous, and the possibility of the isolated material being an artifact could not therefore be excluded. The authors considered the possibility, not specifically mentioned, that epimerization of the C-16 hydroxyl group of oestriol might have occurred to a small extent, either during the preliminary hot acid hydrolysis of the urine, or subsequently through the use of aqueous alkali in the fractionation of the urinary extract. One of the purposes of the work reported here was to investigate this possibility.