The Mechanism of Ficin-Catalysed Reactions

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(Received 27 October 1958)

During the last few years a number of hydrolytic and acyl-transfer enzymes have been studied in detail and marked similarities in their reaction mechanisms have been found. Cholinesterase (Wilson & Calib, 1956), chymotrypsin (Gutfreund & Sturtevant, 1956) and trypsin (Gutfreund, 1955*a*) have been shown to react with their substrates by a three-step mechanism involving an initial rapid adsorption of the substrate and a subsequent chemical reaction with an active site consisting of a histidine imidazole and a serine hydroxyl group. More recent studies on phosphoglucomutase (Koshland & Erwin, 1957) and on thrombin (Gladner & Laki, 1958) revealed that these enzymes also have serine hydroxyl groups as a component of their active sites. Gutfreund (1955*b*) and Bernhard & Gutfreund (1956) proposed a catalytic mechanism for ficin which is almost identical with that proposed for papain-catalysed reactions by Kimmel & Smith (1957). This mechanism again involves an initial adsorption process and a subsequent chemical reaction with the catalytic groups. The catalytic site of ficin contains a sulphydryl group which is acylated during the reaction between enzyme and substrate. This paper describes experiments which were carried out to extend the studies of the properties and reactivity of the active site of ficin, which were begun by Bernhard & Gutfreund (1956).

EXPERIMENTAL

Methods

The constant-pH titration procedure as adapted by Hammond & Gutfreund (1955) was used to follow the rate of enzyme-catalysed ester hydrolysis reactions. Standard conditions for the assay of ficin activity are: temperature, $25 \pm 0.05^\circ$; 15 ml. of reaction mixture of the following composition: benzoyl-L-arginine ethyl ester, 13.7 mM; NaCl, M; ethylenediaminetetra-acetic acid (EDTA; mM). Appropriate volumes of enzyme are included in 15 ml. of reaction mixture. The rate of hydrolysis can be expressed as moles of acid produced/sec./mole of enzyme.

For the measurement of amide hydrolysis it was found that the photometric ninhydrin method of Moore & Stein (1954) gave sufficient sensitivity and accuracy to allow the determination of initial rates when only small quantities of ammonia had been produced.

Materials

N-Benzoyl-L-arginine ethyl ester (BAEE) and *N*-benzoyl-L-arginine amide (BAA) were prepared from L-arginine monohydrochloride (British Drug Houses Ltd.) after the method of Bergmann, Fruton & Pollock (1939).

BAA gave the following figures on analysis [the figures in parentheses are those calculated for the formula BAA, $\text{HCl} \cdot \text{H}_2\text{O}$ (*M*, 332)]: N = 20.4% (21.1), Cl = 9.8% (10.7), H_2O = 5.8% (5.7).

BAEE is a hygroscopic substance associated with varying amounts of water. Solutions of this substrate were analysed by trypsin hydrolysis. Assays were generally 95% of the value estimated from the known weight of substrate and a molecular weight of 343, based on the formula BAEE.HCl. The chief impurity was water.

N-Benzoylglycine methyl ester (hippuric methyl ester) was prepared from hippuric acid (British Drug Houses Ltd., London) by the ion-exchange method. IR-120 (H) resin (British Drug Houses Ltd., London) was freed from colouring matter by refluxing with methanol before use. Dried hippuric acid (5 g.) was suspended in 200 ml. of dry methanol and refluxed for 12 hr. with 5 g. of the resin. Finally the resin was filtered off and the methanol removed under vacuum. The ester was recrystallized from aqueous methanol in approx. 80% yield. This method is unsuitable for positively charged amino acid derivatives since the resin is positively charged.

Hippuric amide was prepared by saturating a solution containing 5 g. of hippuric methyl ester in 200 ml. of dry methanol with ammonia gas under anhydrous conditions. After storage of the resulting solution at room temperature for 2 days methanol was removed under vacuum until crystallization commenced. The amide was recrystallized from aqueous methanol with approx. 75% yield.

Ficin was obtained as the dried latex from L. Light and Co. Ltd., Colnbrook, Bucks. In this form the enzyme was found to be stable for periods of over 1 year, judged by the constant specific activity obtained with different preparations during this time interval. The preparation was done in a cold room at 4° . Crude latex (20 g.) was suspended in 200 ml. of 0.01N-HCl (resulting pH 4.0) and stirred for 2 hr. The resulting suspension was then dialysed with stirring against 5 l. of mM-EDTA (pH 4.4) for 15 hr. The insoluble material was removed by centrifuging and the resultant enzyme solution [(1) of Table 1] was purified by ammonium sulphate fractionation, details of which are shown in Table 1. After each precipitation the suspension of precipitate was dialysed against mM-EDTA (pH 4.4) and the volumes indicated are those of the material redissolved in this manner. Except for pilot experiments fraction (8) was used for kinetic measurements.

The protein concentrations given in Table 1 were determined by measurement of extinction at 279 m μ after a 50-fold dilution of the enzyme solution. A calibration curve relating extinction to Kjeldahl nitrogen enabled the easy evaluation of protein concentration, by assuming that the protein contained 16.0% of Kjeldahl nitrogen. An extinction of 1 corresponded to protein concentration of 2.2%. The specific enzymic activities listed in column four have been converted into maximum velocities of hydrolysis for 0.1 ml. of a 1% protein solution, with the value of K_m for BAEE determined later.

In order to obtain ficin preparations which were fully active without the addition of a reducing substance, it was found necessary to work as rapidly as possible at low temperature (4°). Such preparations were required for work with organic mercury inhibitors and for kinetic investigations in alkaline solution.

RESULTS

Fully active ficin preparations in the absence of the activator show stability in the range pH 3.5–9.0. This is based on the immediate recovery of full activity at the pH optimum after storage at the extremes of pH for times of up to 60 min. at 25°. Fully active preparations show no enhancement of activity on the addition of 2 mm-2:3-dimercaptopropanol when first prepared, but slowly lose activity on storage at 4°. After 6 weeks the activity is approximately one-half of its initial value. It is likely that such a change is caused by

oxidation of thiol groups to the disulphide form, possibly catalysed by trace-metal impurities in the manner suggested by Lamfrom & Nielson (1957). This oxidation was found to be completely reversed by the addition of dimercaptopropanol.

The addition of mercury compounds to ficin solutions (fully active without reducing substances) destroys enzymic activity. The activity is, however, completely restored by 2 mm-2:3-dimercaptopropanol. This indicates that the loss of enzymic activity is not due to denaturation of the protein. By titration of ficin activity with methylmercuric hydroxide it was found that 1 mole of this inhibitor completely inactivated 26 kg. (or 1 mole) of enzyme (Bernhard & Gutfreund, 1956). These results suggest strongly that a thiol group of the enzyme is essential for its activity and that there is only one group/enzyme molecule.

Variation of substrate and substrate concentration

Ficin-catalysed hydrolyses of BAEE, BAA and hippuric methyl ester were found to obey accurately Michaelis-Menten kinetics. The observed velocity of hydrolysis was found to be proportional to the enzyme concentration over a 25-fold range.

The main limitation of the accuracy of the Michaelis parameter determinations with Eadie (1942) plots was the maximum solubility of substrate relative to K_m . Table 2 lists the values of the

Table 1. Assays of N-benzoyl-L-arginine ethyl ester relating to ficin preparation and description of successive operations

Assay number and description of successive operations	Protein (%)	Total amount of protein (g.)	10 ³ × Specific esterase activity (moles/sec./0.1 ml. of 1% protein)
(1) Original solution (195 ml.)	2.28	4.44	4.9
(2) 0.5 Saturated ammonium sulphate precipitate (158 ml.)	1.90	3.00	5.5
(3) Supernatant (300 ml.)	0.47	1.41	0.84
(4) 0.3 Saturated ammonium sulphate precipitate (120 ml.)	1.42	1.71	9.0
(5) Supernatant (158 ml.)	0.69	1.09	2.3
(6) 0.5 Saturated sodium chloride precipitate (100 ml.)	0.81	0.81	9.7
(7) Supernatant (116 ml.)	0.75	0.87	5.0
(8) 0.5 Saturated sodium chloride precipitate (100 ml.)	0.49	0.49	9.7
(9) Supernatant (98 ml.)	0.31	0.30	8.3

Table 2. Michaelis parameters for the ficin-catalysed hydrolyses of N-benzoyl-L-arginine ethyl ester, N-benzoyl-L-arginine amide, hippuric methyl ester and hippuric amide

Results were obtained at 25° and pH 6.06 in the presence of mm-EDTA buffer and m-NaCl. For BAEE under these conditions $V_{max} = 9.6 \times 10^{-8}$ moles/sec./0.1 ml. of 1% enzyme solution.

Substrate	BAEE	BAA	Hippuric methyl ester	Hippuric amide
10 ³ × Concentration range (M)	0.5–5.0	1.0–8.0	0.25–3.25	0.40–3.0
10 ³ K_m (M)	2.5 ± 0.3	4.8 ± 0.5	4.8 ± 1.0	13 ± 3
V_{max} (relative values)	1.0 ± 0.1	0.9 ± 0.1	1.4 ± 0.2	0.04 ± 0.01

Michaelis parameters of the various substrates at pH 6.06 and 25°. The table also includes the range of substrate concentrations used for each determination.

Effect of pH

The stability of ficin under different conditions of pH and activator concentration has already been mentioned. Fig. 1 shows the pH-dependence of the observed rates of hydrolysis of four substrates.

For the esterase runs at low pH, where the products were incompletely ionized, a small percentage of the product did not produce hydrogen ions. Such hydrolysis rates were corrected by using pK values for benzoyl-L-arginine or benzoyl-L-glycine.

Only esterase runs were attempted in alkaline solution because of the difficulties of working in a carbon dioxide-free atmosphere. All measurements

at alkaline pH were performed under an atmosphere of carbon dioxide-free nitrogen. A blank run was performed which furnished a correction for the spontaneous hydrolysis of the substrate, with possibly a small contribution from absorption of carbon dioxide. Such corrections were only a few per cent of the measured enzymic rates.

The full line in Fig. 1 is the theoretical curve for the ionization of two groups of the enzyme (pK_B 4.40 and pK_A 8.46) and indicates how closely the experimental points fit an ionization curve.

K_m measurements at 25° for the substrates BAEE and BAA at the pH specified are given in Table 3. This is to indicate the accuracy of the measurements. There may be a slight increase in K_m at pH 4.04, but in view of the reproducibility of the measurements this is barely significant.

The variation of ficin activity with pH must be interpreted in terms of ionizations of two groups on the enzyme, as the substrates used do not change their states of ionization in this pH range.

The relevant equation for the variation of enzymic activity in terms of the ionization of both a basic and an acidic group is

$$\frac{V^0}{V^1} = 1 + \frac{[H^+]}{K_B} + \frac{K_A}{[H^+]},$$

V^0 is the rate of hydrolysis at the optimum pH and V^1 is the rate of hydrolysis at other pH values. The subscripts of the ionization constants, K_A and K_B , indicate whether the ionizing group is a Brønsted acid or base at the pH optimum. The K_A term is dominant at low concentrations of hydrogen ion and the K_B term is dominant at high concentrations of hydrogen ion.

Fig. 2 shows the data of Fig. 1 for BAEE and hippuric methyl ester plotted in terms of the above equation. In acid solution the graph of V^0/V^1 versus $[H^+]$ has a slope $1/K_B$ and in alkaline solution the graph of V^0/V^1 versus $1/[H^+]$ has a slope K_A . The two pK values for each substrate at 25° are shown on the graph.

Influence of ionic strength

Ficin action has been found to depend markedly upon salt concentration. Fig. 3 shows the variation of the observed velocity of hydrolysis at low ionic strengths for the substrates BAEE, BAA and hippuric methyl ester. Different salts were used to investigate whether the effect was due to ionic strength.

It was not possible to work at ionic strengths below 0.01M (the substrate concentration employed) because the arginine substrates themselves make a contribution to the ionic strength. It was not possible to decide whether such an effect was due to changes in one of the Michaelis parameters or in both.

Fig. 1. Variation of observed rates of ficin-catalysed hydrolysis with pH for the substrates O, BAEE, ●, hippuric methyl ester, △, BAA and ▲, hippuric amide. Substrate, 0.02M; NaCl, 0.01M. Results are expressed as percentages of their respective rates at pH 6.5, so that they can be presented as one graph.

Table 3. pH-Dependence of K_m values for the action of ficin on N-benzoyl-L-arginine ethyl ester and N-benzoyl-L-arginine amide

Results were obtained at 25° in mM-EDTA and 0.1M-NaCl.

Substrate	pH	$10^2 K_m$ (M)
BAEE	8.43	2.1
	6.33	2.5
	6.04	2.8
	6.04	2.4
	6.04	2.6
	6.04	2.7
	6.04	2.3
	5.34	2.0
	4.63	2.3
	4.63	2.3
	4.63	2.2
	4.04	3.0
	4.04	3.3
	6.30	5.2
BAA	6.08	4.4
	5.15	4.1
	4.33	4.5

At high ionic strengths, however, the results of Table 4 suggest that any change in the observed velocity of hydrolysis is due to changes in $V_{\max}$ and that K_m is almost constant. Fig. 4 presents the results of an investigation on the effects of different salts on the hydrolysis of BAEE at high ionic strengths.

Fig. 2. Determination of the dissociation constants, K_A and K_B , of groups on the enzyme from the data of Fig. 1 and Table 3 for the pH dependence of the hydrolysis of BAEE (●) and hippuric methyl ester (○).

Fig. 3. Variation of observed velocity of ficin-catalysed hydrolysis of BAEE, BAA and hippuric methyl ester at low ionic strengths; pH 6.04, mM-EDTA, 0.01M-substrate. BAEE-NaCl, ○; BAA-NaCl, △; hippuric methyl ester-NaCl, ●; BAEE-Na₂SO₄, □.

Influence of propan-2-ol

The effect of propan-2-ol on the ficin-catalysed hydrolysis of BAEE, BAA and hippuric methyl ester was studied at pH 6.3 and 25.0° with mM-EDTA as buffer and in the absence of added sodium chloride. The maximum volume concentration of propan-2-ol used was 33% (approx. 4.3M) and no denaturation of enzyme occurred under these conditions, as judged by the complete recovery of enzymic activity on dilution.

The results obtained for the inhibition of ficin action by propan-2-ol do not fit the usual equations for competitive or non-competitive inhibition. The variation of the Michaelis parameters for BAEE, BAA and hippuric methyl ester with propan-2-ol concentration is given in Table 5.

For all substrates the presence of propan-2-ol causes a marked increase in K_m . The influence of propan-2-ol concentration on $V_{\max}$ for the charged substrates BAEE and BAA is also very similar. For the uncharged substrate hippuric methyl ester, however, there does not appear to be any significant change in $V_{\max}$ with increasing

Table 4. Effect of sodium chloride concentration on K_m and $V_{\max}$ for the action of ficin on N-benzoyl-L-arginine ethyl ester

Results were obtained at 25° and pH 6.04 in mM-EDTA. In the presence of M-NaCl, $V_{\max} = 9.6 \times 10^{-8}$ M/sec./0.1 ml. of % ficin.

[NaCl] (M)	$10^3 K_m$ (M)	$V_{\max}$ (relative values)
0.05	2.5	1.0
0.50	2.5	1.1
1.00	2.7	1.1
1.70	2.8	1.5

Fig. 4. Effect of different salts on the ficin-catalysed hydrolysis of BAEE; pH 6.04, mM-EDTA, 0.01M-substrate. NaCl, ○; KCl, ●; NH₄Cl, □; KBr, ■; KI, ×; NaNO₃, △; KNO₃, ▲; KSCN, +.

Table 5. *Effect of propan-2-ol concentration on K_m and V_{max} for the action of ficin on N-benzoyl-L-arginine ethyl ester, N-benzoyl-L-arginine amide and hippuric methyl ester at specified pH and 25°*

mm-EDTA buffer was used, in the absence of added sodium chloride.

Substrate and pH	Concn. of propan-2-ol (M)	$10^3 K_m$ (M)	V_{max} (relative) (%)
BAEE, 6.04	0	2.5	100
	0.87	4.6	76
	1.74	6.5	54
	2.63	6.7	29
BAA, 6.30	0	5	100
	1.31	11	68
	2.62	45	23
Hippuric methyl ester, 6.34	0	5	100
	0.87	9	100
	2.62	25	100

Fig. 5. Effect of propan-2-ol concentration (○, 2.62M; ●, 0.87M) on the ficin-catalysed hydrolysis of hippuric methyl ester at pH 6.04 in mm-EDTA. Δ, No propan-2-ol.

propan-2-ol concentration. The evidence for this is shown in Fig. 5, where Eadie (1942) plots for the determination of the Michaelis parameters at specified propan-2-ol concentrations are shown. The slope of an Eadie plot is $1/V_{max}$, and the ordinate intercept is K_m/V_{max} .

It is difficult to evaluate the Michaelis parameters accurately in this case because the solubility of hippuric methyl ester is low compared with the value of K_m to be determined. A decrease in V_{max} with increasing propan-2-ol concentration would require an increase in slope of the Eadie plots. The experimental results do not support this. The lines drawn through the experimental points in Fig. 5 have a constant slope which would imply that V_{max} is unaffected by propan-2-ol for this substrate. The degree to which the experimental results support this proposal may be gauged from the Figure. This relative invariance of V_{max} for hippuric methyl ester may be compared with the change in V_{max} observed with BAEE and BAA at corresponding propan-2-ol concentrations (Table 5). There is, however, an increase in K_m for hippuric methyl ester and this is illustrated by the increase in the ordinate intercept in Fig. 5 with increasing propan-2-ol concentration.

Effect of temperature

The variation of the Michaelis parameters K_m and V_{max} , in the temperature range 10–40°, has been investigated for the substrates BAEE, BAA and hippuric methyl ester. The ester substrates were studied at two pH values; the amide was studied in the region of the pH optimum only. The results are set out in Table 6.

Table 6. *Variation of the Michaelis parameters with temperature and the derived Arrhenius activation energy*

mm-EDTA was used as buffer throughout. Different convenient amounts of enzyme were used for each substrate: BAEE experiments, 13.3×10^{-8} moles of enzyme; BAA experiments, 11.5×10^{-8} moles of enzyme; hippuric methyl ester (HME) experiments, 5.06×10^{-8} moles of enzyme.

Substrate and pH	Temp. (°K)	V_{max} (arbitrary units)	E_a (kcal./mole)	$10^3 K_m$ (M)
BAEE, 6.25	285.0	11.6	—	1.9
	298.0	33.2	12.8	2.5
	312.5	85.6	—	3.3
BAEE, 4.60	285.0	6.6	—	—
	298.0	19.6	14.0	—
	312.5	58.8	—	—
HME, 6.25	287.7	9.0	—	3.9
	297.9	17.7	10.4	4.8
	312.8	38.6	—	6.5
HME, 4.25	287.7	5.2	—	—
	297.9	9.1	11.0	—
	312.8	24.0	—	—
BAA, 6.25	284.2	9.1	—	4.3
	298.6	25.8	11.6	4.8
	313.7	62.4	—	5.5

Fig. 6 is a graph of the data for hippuric methyl ester to determine the activation energy, E_a , by means of the usual Arrhenius equation

$$\log k = \frac{-E_a}{2.303 RT} + \text{constant}.$$

This relationship was found to be linear for all substrates in the temperature range 10–40°, within experimental error.

DISCUSSION

The results of detailed kinetic investigations of the ficin-catalysed hydrolysis of a number of esters and amides give information about the physicochemical properties of those groups on the enzyme molecule which can be called the catalytic groups. From these properties one can make deductions about the probable identity of these groups and about the mechanism of the reactions which they undergo with the substrates.

A number of different methods have been used to determine the dissociation constants of groups essential for the reactivity of enzyme-substrate compounds. Our previous studies on trypsin (Gutfreund, 1955*a*) and chymotrypsin (Hammond & Gutfreund, 1955) encouraged us to use the simple method of interpreting plots of reaction rate against pH as titration curves to get a first picture of the situation. Fig. 1 shows that one acidic and one basic group are involved in the reactivity of ficin. The pK values for the two groups are evaluated from the plots in Fig. 2. Comparisons of pK values obtained for groups on protein surfaces with known constants for free amino acids have to be treated with caution. For the characterization of active groups one must consider both the general variations of pK values with charge configuration and dielectric properties of the surroundings and

the special circumstances which are responsible for the peculiar reactivity of groups on the active site; this point is elaborated below. The acidic group is most likely to be a carboxyl. The basic group cannot be identified from its pK alone, but it will be shown below that there is good evidence for a positively charged group on the active site. It can therefore be concluded that an NH_3^+ group is involved. None of the substrates used has groups which change their ionization over the pH range examined here.

It has been shown previously (Bernhard & Gutfreund, 1956) that a sulphydryl group is required for the activity of the enzyme. This has been confirmed during the present studies by titration with methylmercuric hydroxide and subsequent reactivation by dimercaptopropanol.

Fig. 3 shows that the effect of variation of ionic strength (I) in the observed velocity of hydrolysis at low values of I is different for charged and uncharged substrates. The line drawn through the experimental points for BAEE and BAA in Fig. 3 has the theoretical slope for a uni-univalent-like charge interaction with the well-known Debye-Hückel-Brönsted equation. As the arginine substrates have unit positive charge at pH 6.0 these results suggest that there is a positively charged group at the active site of the enzyme. There is a similar enhancement of observed velocity of hydrolysis for charged and uncharged substrates at concentrations of sodium chloride greater than approximately 0.15M.

Propan-2-ol is a suitable solvent for studies of dielectric effects on enzyme systems. Although a large variety of complex changes can occur with altered solvent composition, the data presented in Figs. 5 and 7 are readily interpreted as a dielectric effect on the charge interaction in the enzyme-substrate compound. Propan-2-ol molecules are no doubt adsorbed on the enzyme by van der Waals' forces and hydrogen bonds. This would hinder the adsorption of the substrate at the active site and the formation of the Michaelis-Menten complex. It is possible to explain the increased K_m values in the presence of propan-2-ol in this way. On a molar basis propan-2-ol is a better inhibitor than ethanol, presumably because of its greater van der Waals' attraction to the protein. The invariance of V_{max} with propan-2-ol concentration for the uncharged substrate hippuric methyl ester compared with the decrease observed for the charged substrates BAEE and BAA suggests that the last-mentioned effect is most likely due to the repulsion between the two positive charges on the substrate and active site. The variation of V_{max} for BAEE hydrolysis with propan-2-ol concentration is presented in Fig. 7 in terms of the theoretical relationship between the log of the rate constant

Fig. 6. Analysis of data for temperature-dependence of the ficin-catalysed hydrolysis of hippuric methyl ester (see Table 6); mM-EDTA; M-NaCl; ●, K_m at pH 6.25; Δ, V_{max} at pH 6.25 and ○, V_{max} at pH 4.25.

and the reciprocal of the dielectric constant of the medium (Scatchard, 1932). A linear relationship is obtained and its slope enables the calculation of the distance of closest approach of the arginine side chain and the active site, assuming that the interaction is between two unit positive charges. The value of $1.3\text{ \AA}$ so obtained is small but of a reasonable magnitude for the approximations involved.

Two important conclusions can be drawn from the investigations of the effect of temperature on the ficin-catalysed hydrolysis of esters and amides. First it can be seen in Table 6 that the difference between the energy of activation at a pH near the pK of the acidic group and at pH 6.25 is small. One can derive from this that the heat of ionization of the acidic group is small (Gutfreund, 1955*a*), which would confirm that a carboxyl ionization is involved. The second point of interest is demonstrated by the fact that the energies of activation for the ficin-catalysed hydrolysis of esters and amides are very similar. [For the trypsin-catalysed hydrolysis of the same pair of ester and amide substrates considerably different energies of activation were found (Schwert & Eisenberg, 1949; Butler, 1941).] This confirms an earlier conclusion from similarity of rate of hydrolysis (Bernhard & Gutfreund, 1956) that the rate-determining step for the two substrates is the same. Since esters and amides have such widely different characteristics towards hydrolytic attack as demonstrated, for example, by the oxygen-exchange experiments of Bender, Gringer & Kemp (1954), it can only be concluded that the rate-determining step is the decomposition of an intermediate common to the reaction of ficin with BAEE and BAA. It seems reasonable to suggest that this common intermediate is an enzyme acylated by the substrate at the sulphydryl

group. The reaction path of ficin (E) with BAA or BAEE (AB) can then be written:

where EAB is a rapidly formed loose enzyme-substrate compound; the formation of the acyl enzyme EA and its decomposition to free enzyme and acid can be regarded as irreversible while product concentrations are low. From the discussion above it is concluded that the step characterized by k_3 is rate-determining and corresponds to the turnover number or $V_{\max}$ of the enzyme. The step characterized by k_2 (the acylation of the enzyme) must be slower for an amide substrate than for an ester. It was stated above that the similarity of overall rate of hydrolysis and energies of activation of the reactions with BAA and BAEE indicates the existence of a common intermediate. The coincidence of the dependence of the rate of reaction of the two substrates on pH, temperature and ionic strength is, however, not sufficiently exact to exclude the possibility that for BAA k_2 and k_3 are of the same order of magnitude. Some comments must be made on the relative rates of hydrolysis of hippuric methyl ester and hippuric amide. The fact that $V_{\max}$ for hippuric methyl ester is somewhat larger than for BAEE indicates that the decomposition of hippuryl acylated enzyme is somewhat faster than that of the benzoyl-L-arginyl acylated enzyme. The very much slower rate of hydrolysis of hippuric amide indicates that for this substrate the formation of the acyl enzyme is rate-determining. The low solubility of the uncharged substrates and the large K_m for hippuric amide make accurate calculation of constants for this substrate difficult.

One of us (Gutfreund, 1955*b*) has attempted to determine k_1 for the reaction of ficin with BAEE by the 'initial acceleration' method. The value of $500\text{ M}^{-1}\text{ sec}^{-1}$ was assigned to k_1 because it was shown to be a second-order constant and only the formation of the first enzyme-substrate compound is a second-order process. It can, however, be shown from the analysis of the three-step process used to describe the reaction of this and other hydrolytic enzymes (Gutfreund & Sturtevant, 1956) that if, at substrate saturation, $[EAB] \ll [EA]$ the rate of formation of EA (the rate-determining enzyme-substrate compound) will be

$$\frac{d[EA]}{dt} = k_2[EAB],$$

and if the steady-state concentration of $[EAB]$, which is very small compared with the total enzyme concentration, is reached rapidly, then the pseudo-second-order constant quoted above is an evaluation of $k_2[EAB]$.

Fig. 7. Variation of $\log V_{\max}$ with dielectric constant (ϵ) for the ficin-catalysed hydrolysis of BAEE (pH 6.04 in mM-EDTA (see Table 5)).

We have in this manner obtained good evidence for the chemical nature of the catalytic site and for the relative rates of the three distinguishable steps in the formation and decomposition of the enzyme-substrate compounds of ficin. The reaction mechanism of steps involved in the interaction, between the three groups CO_2^- , NH_3^+ and SH on the enzyme surface and the carboxyl group of different substrates, is open to a number of interesting speculations. We should like to propose the steps shown in Scheme 1 for the reaction between enzyme and substrate.

Bender (1957) has described some intramolecular-catalysis reactions which would simulate such steps in enzymic reactions. Stockell & Smith (1957), Smith, Chavré & Parker (1958) and Kimmel & Smith (1957), who have undertaken a detailed analysis of the reactions and properties of the closely similar enzyme papain, have proposed a somewhat different reaction mechanism. They assign the $\text{pK } 8.4$ to the catalytic $-\text{SH}$ group and assume that the enzyme cannot be acylated by the substrate when the sulphhydryl group is ionized. For ficin we object to this interpretation on two grounds. First, slowing down of the acylation by partial ionization of $-\text{SH}$ should not affect the overall rate since the hydrolysis of the acyl enzyme is rate-determining. Secondly, this mechanism

does not involve the positive group known to participate in the reaction. Our interpretation involves both ionized $-\text{CO}_2^-$ and NH_3^+ groups in the rate-determining decomposition of the acyl enzyme.

Finally it should be pointed out that the relative stability of the acyl enzyme compound accounts for the efficiency of the ficin- and papain-catalysed acyl transfer to acceptors other than water, as in transpeptidation. This is discussed by one of us elsewhere (Gutfreund, 1957) in quantitative terms.

SUMMARY

1. The kinetics of ficin-catalysed hydrolyses of benzoyl-L-arginine ethyl ester, methyl hippurate and of the corresponding amides was studied under a variety of conditions of temperature, pH, ionic strength and dielectric constant.

2. From the results of these experiments it can be concluded that three reactive groups are necessary for the catalytic action of ficin; these are $-\text{SH}$, NH_3^+ and CO_2^- .

3. A reaction sequence between enzyme and substrate is proposed which involves the rapid formation of a loose enzyme-substrate compound, a subsequent acylation of the enzymic $-\text{SH}$ group by the carbonyl of the substrate and finally the decomposition of the acyl enzyme.

4. Reaction mechanisms for the second and third step can be formulated by analogy with homogeneous bifunctional catalysis.

One of us (B.R.H.) is indebted to the Ramsay Memorial Trustees for the award of a Fellowship. We should also like to record our appreciation of the most helpful and stimulating advice we have received from Dr E. L. Smith at various times.

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The Effects of Adenosine Triphosphate on the Mechanical Properties of Surface Films of L-Myosin and Actomyosin

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(Received 20 October 1958)

The earliest study of the effects of the adenine nucleotides on the properties of the myofibrillar proteins spread at the air-water interface was made by Munch-Petersen (1948), who found that when actomyosin was spread on 0.5 M-potassium chloride solution containing 0.1 mM-adenosine triphosphate, it occupied an area considerably greater, for a given surface pressure, than in the absence of adenosine triphosphate. The effect was later shown to be due, at least in part, to an increase by adenosine triphosphate in the rate of spreading of the protein (Lajtha & Rideal, 1952; Cheesman, 1952). Similar effects were found for actin-free myosin by Munch-Petersen, although Cheesman was unable to repeat this observation.

The acceleration by adenosine triphosphate of the spreading of actomyosin may be explained by the dissociation of the protein by this nucleotide into its constituent actin and myosin. A similar acceleration is produced by high salt concentrations, which also dissociate actomyosin in bulk

solution. For this reason, the results seemed to be of little biochemical interest, especially as no evidence of chemical alteration of the protein by adenosine triphosphate could be obtained from phase-boundary potential measurements (Cheesman, 1952).

Cheesman & Sten-Knudsen (1959) have developed a procedure for the study of the viscoelastic properties of protein films. It was found with this method that when actomyosin or L-myosin was spread on M-potassium chloride solution containing 0.1 mM-adenosine triphosphate, the mechanical rigidity of the resulting film was considerably lower than that of a similar film in the absence of adenosine triphosphate at the same surface pressure with the first protein, and at the same surface pressure and specific area with the second. Two explanations of these findings presented themselves. It was conceivable that a complex of adenosine triphosphate and denatured protein persisted in the spread film, with a consequent effect upon the mechanical properties of the latter. Alternatively, since the protein under the conditions of the experiments was not fully spread, the effect could have been due to an interaction between adenosine triphosphate and native

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