Papain-Catalysed Hydrolysis of some Hippuric Esters

A NEW MECHANISM FOR PAPAIN-CATALYSED HYDROLYSES

By G. LOWE AND A. WILLIAMS

The Dyson Perrins Laboratory, University of Oxford

(Received 30 October 1964)

1. The Michaelis-Menten parameters for the papain-catalysed hydrolysis of a number of alkyl, aryl and alkyl-thiol esters of hippuric acid have been determined. 2. For all the aryl esters and most of the alkyl esters studied, the catalytic constant, k_0 , is $2-3 \text{ sec.}^{-1}$ and most probably represents deacylation of the common intermediate, hippuryl-papain. 3. Two alkyl esters and hippurylamide, however, have catalytic rate constants, k_0 , less than $2-3 \text{ sec.}^{-1}$. It is possible to interpret all the available kinetic data in terms of a three-step mechanism in which an enzyme-substrate complex is first formed, followed by acylation of the enzyme through an essential thiol group, followed by deacylation of the acyl-enzyme. 4. The logarithm of the ratio of the Michaelis-Menten parameters, which reflect the acylation rate constant, for four aryl esters of hippuric acid studied give a linear Hammett plot against the substituent constant, σ . Arguments are presented that indicate acid as well as nucleophilic catalysis in the acylation process and that the most likely proton donor is an imidazolium ion. 5. It is suggested that this imidazolium ion is part of the same histidine residue that has been tentatively implicated in the deacylation process (Lowe & Williams, 1965b). 6. A new mechanism is proposed for the papain-catalysed hydrolysis of *N*-acyl- α -amino acid derivatives.

The extensive studies of Smith and his collaborators on the proteolytic enzyme, papain, led them to postulate a two-step mechanism for the hydrolysis of *N*-acyl-amino acid derivatives (Smith, 1958). The first step involved the acylation of the enzyme through an essential thiol group, whereas in the second step deacylation of the acyl-enzyme occurred. The pH-rate profile for the acylation step in the papain-catalysed hydrolysis of *N*- α -benzoyl-L-arginine ethyl ester was shown to be bell-shaped, and, from the half-maximal rate, two groups with apparent pK_a values 4.3 and 8.02 at 37° were implicated (Smith & Parker, 1958). The group with apparent pK_a 4.3 showed negligible heat of ionization and was attributed to a carboxylate ion. The group with apparent pK_a 8.02 showed a heat of ionization 5.1 kcal./mole at 0° and was attributed to a thiol group, although the conjugate acid of an amino or imidazole group could not be excluded. The reason the thiol group was favoured was because such a group was known from inhibition experiments to be essential for enzymic activity.

We chose to study the papain-catalysed hydrolysis of a series of hippuric esters to establish the true deacylation constant for hippuryl-papain that was required for the work on the mechanism of deacylation (Lowe & Williams, 1965b). By studying

a series of aryl esters of hippuric acid it was hoped to learn more about the mechanism of the acylation process.

MATERIALS AND METHODS

Preparation of substrates. Five general methods of ester formation were used. The successful method employed for each substrate is indicated in Table 1.

(a) Fischer-Speier. Dry HCl was passed into a refluxing solution of hippuric acid (2g.) in the alcohol (50 ml.) for 2 hr. The solution was left for 12 hr. and concentrated under reduced pressure. Cooling to -15° yielded crystals that were purified by recrystallization from a suitable solvent.

(b) Ion-exchange resin (cf. Hammond & Gutfreund, 1959). A solution of hippuric acid (5g.) in the alcohol (20 ml.) and benzene (75 ml.) was refluxed with an ion-exchange resin (Amberlite IR-120, 2g., that had been treated with 2*N*-HCl and washed thoroughly with the alcohol) in a Dean and Stark apparatus for approx. 6 hr. until the calculated amount of water had accumulated in the measuring tube. The ester was recovered by concentrating the filtered solution under reduced pressure. Cooling the residue to -15° yielded crystals that were purified by recrystallization.

(c) Acid chloride (cf. Johnston, 1956). Hippuryl chloride (2g.) was added to the alcohol (1g.) and cooled to 0°. Pyridine (0.8g.) was then slowly added and the mixture left for $\frac{1}{2}$ hr. The product was acidified and extracted with ether. The ethereal layer was dried (over Na_2SO_4) and

Table 1. *Melting points and analytical data of hippuric esters*

Yields were greater than 90%. Details of methods (a)–(e) are given in the Materials and Methods section. References: ¹, Hammond & Gutfreund (1959); ², Epan & Wilson (1963); ³, Weiss (1893); ⁴, McDonald & Balls (1957); ⁵, Franzan (1909); ⁶, Lillivek & Sandstrom (1954); ⁷, Johnston (1956); ⁸, Schwyzer (1958).

Ester (hippurate)	m.p.	Literature m.p.	Method	Analyses				
					C(%)	H(%)	N(%)	Cl(%)
Methyl	81°	80–81.5 ¹	(a)	Found	62.0	5.6	7.4	
				Calc. for C ₁₀ H ₁₁ NO ₃	62.1	5.7	7.3	
Ethyl	59–60	60.5 ^{1,5}	(b)	Found	63.8	6.2	6.7	
				Calc. for C ₁₁ H ₁₃ NO ₃	63.9	6.3	6.8	
<i>n</i> -Propyl	58–59	New	(b)	Found	65.4	6.9	6.5	
				Calc. for C ₁₂ H ₁₅ NO ₃	65.2	6.8	6.3	
Isopropyl	87–88.5	85.5–86.5 ^{1,2}	(b)	Found	64.9	6.8	6.2	
				Calc. for C ₁₂ H ₁₅ NO ₃	65.2	6.8	6.3	
<i>n</i> -Butyl	30–32	33–35 ^{1,6}	(b)	Found	66.5	7.3	6.2	
				Calc. for C ₁₃ H ₁₇ NO ₃	66.4	7.2	6.0	
Thioethyl	88	85–86 ⁷	(c)	Found	59.0	5.9	6.4	14.5
				Calc. for C ₁₁ H ₁₃ NO ₂ S	59.2	5.9	6.3	14.5
2-Carboxythioethyl	142–143	144 ⁸	(d)	Found	53.7	5.1	5.1	11.8
				Calc. for C ₁₂ H ₁₃ NO ₄ S	54.0	4.9	5.3	12.0
Phenyl	104	104 ³	(e)	Found	71.1	5.2	5.2	
				Calc. for C ₁₅ H ₁₃ NO ₃	70.8	5.1	5.5	
<i>m</i> -Nitrophenyl	118–122	New	(e)	Found	60.2	3.9	9.2	
				Calc. for C ₁₅ H ₁₂ N ₂ O ₅	60.0	4.0	9.3	
<i>p</i> -Nitrophenyl	170–171	167–168 ^{3,4}	(e)	Found	60.1	4.1	9.3	
				Calc. for C ₁₅ H ₁₂ N ₂ O ₅	60.0	4.0	9.3	
<i>p</i> -Chlorophenyl	130–131.5	New	(e)	Found	62.0	4.4	5.2	12.0
				Calc. for C ₁₅ H ₁₂ ClNO ₃	62.1	4.2	4.9	12.3

evaporated, and the residue gave crystals on cooling to –15°.

(d) Mixed anhydride. Hippuric acid (3.5 g.) was dissolved in chloroform (2 ml.) and triethylamine (2 ml.) and cooled to –15°. The mixture was allowed to come to 0° and a solution of the alcohol (2 g.) in chloroform (10 ml.) and triethylamine (2 ml.) were added. After being kept for 5 min. at 0° the solution was allowed to warm to room temperature in 30 min. The chloroform solution was extracted with 2N-HCl, dried (over Na₂SO₄), filtered and evaporated to a small bulk, when the ester usually crystallized. Recrystallization gave a pure product.

(e) Phosphorus oxychloride (cf. Weiss, 1893). Hippuric acid (2.5 g.), POCl₃ (3 g.) and the phenol (1.8 g.) were warmed on a water bath until the mixture became homogeneous. The product was cooled and swirled with water (approx. 20 ml.); when the oily liquid solidified the water was decanted and the solid treated with 2N-NaOH to extract residual phenol and acid. The solid was purified by recrystallization.

Twice-crystallized papain (British Drug Houses Ltd., Poole, Dorset) was activated by the method of Soejima & Shimura (1961) and was assayed against *N*-α-benzoyl-L-arginine ethyl ester (see Lowe & Williams, 1965a).

Kinetics. (a) Titrimetric method. The papain-catalysed hydrolysis of *O*-alkyl hippurates and *S*-alkyl thiohippurates was studied in a 10 ml. thermostatically controlled cell by using a pH-stat [Radiometer titrator (type TTT1c), titration recorder (type SBR2c) and a syringe burette (type SBU1a), which delivered 0.5 ml. of base at full-scale deflection of the recorder]. The reaction rate was determined

from the recorded plot of 0.02N-NaOH added against time to maintain the pH of the solution at pH 6.0. By determining the initial velocities (less than 5% reaction) at different substrate concentrations (range indicated in the Tables), the Michaelis-Menten parameters (from the breakdown of the initial rate, $v = k_0 [E][S_0] / (K_m + [S_0])$) were determined from a Lineweaver-Burk plot by using the method of least squares on a Ferranti Mercury digital computer.

The procedure in a typical run was as follows. A portion of a stock solution of methyl hippurate (approx. 0.02M in 0.3M-NaCl) was added to the cell together with 0.1M-EDTA (0.1 ml.) and sufficient 0.3M-NaCl to give a total of 7 ml. The cell was placed in a tightly fitting jacket through which thermostatically controlled water was passed (good thermal contact was obtained by placing a drop of water between cell and jacket). The solution was stirred magnetically and the pH brought to 6.0 with concentrated reagents. When thermal equilibrium had been reached, 1 ml. of papain solution (approx. 10 μM in 0.3M-NaCl) was added and the production of H⁺ measured.

(b) Spectrophotometric method. The papain-catalysed hydrolysis of *O*-aryl hippurates was studied in the thermostatically controlled cell of a Unicam SP.500 spectrometer modified so that the transmission at a fixed wavelength could be automatically recorded against time. The reactions were performed in buffered solutions and were complete in 30 min. It was possible to use the total progress curves to obtain the Michaelis-Menten parameters, because of the stability of the enzyme over the short time required for the reaction and the lack of product inhibition at the concentrations involved. A Ferranti Mercury digital computer

calculated by a least-squares programme the polynomial of the third order that best fitted the experimental data:

$$\begin{aligned} \text{Extinction} &= a + bt_1 + ct_1^2 + et_1^3 \\ (\text{Hydrolysis rate})^{-1} &\propto (b + 2ct_1 + 3et_1^2)^{-1} \\ (\text{Substrate concn.})^{-1} &\propto (E_{\infty} - E_1)^{-1} \text{ at } t_1. \end{aligned}$$

The computer then calculated and printed out the reciprocal of the hydrolysis rate and the reciprocal of the substrate concentration. The Michaelis-Menten parameters were determined by a least-squares computer programme, by using the known enzyme concentration and the extinction coefficient of the appropriate phenol.

In a typical run 1 ml. of papain (approx. $10 \mu\text{M}$ in 0.3M-NaCl) was diluted to 10 ml. with phosphate buffer, pH 6.0 (made 0.3M with respect to NaCl), containing 1% (v/v) of 0.1M-EDTA . A 3 ml. sample of the solution (approx. $1 \mu\text{M}$ in enzyme) was equilibrated to 35° in a quartz cell in an Adkins thermostat in the cell compartment of the Unicam SP.500 spectrometer. A solution of the phenyl ester in ethanol (0.06ml.) was added with a micro-syringe and the production of phenol observed at a wavelength where the extinction of the ester was negligible. The wavelength used for each ester was as follows (the extinction coefficient in parentheses being that of the undissociated phenol at that wavelength): phenol, $2700\text{\AA}$ ($\epsilon 1160$); *p*-chlorophenol, $2750\text{\AA}$ ($\epsilon 617$); *m*-nitrophenol, $3550\text{\AA}$ ($\epsilon 1520$); *p*-nitrophenol, $3170\text{\AA}$ ($\epsilon 7300$). Spontaneous hydrolysis of the esters was negligible at the pH used.

Back reaction in the papain-catalysed hydrolysis of methyl hippurate. [^{14}C]Methanol (0.1mg. , 0.03mc.) in water (1ml.) was added to a solution of methyl hippurate (32mg.) in water (5.9ml.) plus EDTA (0.1ml. , 0.1M) and the reaction mixture brought to pH 6.0 in the pH-stat. Active papain solution (0.5ml. to make a final concentration of approx. $10 \mu\text{M}$) was added. After about 2 hr., when the ester was half hydrolysed, iodoacetamide (0.1g.) was added to stop the reaction. The residual methyl hippurate was extracted with chloroform, the solution dried (over Na_2SO_4) and concentrated, and the ester (10.3mg.) precipitated with light petroleum (b.p. $60\text{--}80^\circ$). The ester was counted as an 'infinitely thick film' of 0.33cm.^2 cross-sectional area by an end-window Geiger-Müller tube. The time required for 100000 counts was measured for this and subsequent

determinations. The ester (10.3mg.) was diluted with methyl hippurate to 51.5mg. and the material recrystallized to constant specific activity ($1.06\text{m}\mu\text{C/mg.}$). The total activity incorporated into methyl hippurate was therefore $54.5\text{m}\mu\text{C}$.

DISCUSSION

The significance of the Arrhenius parameters for the catalytic constant for methyl hippurate hydrolysis by papain (Table 2) was discussed by Lowe & Williams (1965b).

The most striking feature of the kinetic constants for the papain-catalysed hydrolysis of the hippuric esters is (with the exception of the *n*-butyl and isopropyl esters) the essentially constant value of k_0 (Table 3). The most likely explanation of this effect is that a common intermediate is involved, and this is most likely to be the acyl-enzyme. The same suggestion was made by Smith & Parker (1958), who observed that the catalytic constant, k_0 , for the papain-catalysed hydrolysis of *N*- α -benzoyl-L-arginine ethyl ester and *N*- α -benzoyl-L-argininamide were similar. This being so, the observed catalytic constant, k_0 , represents deacylation of the common acyl-enzyme.

Table 2. *Temperature-dependence of the papain-catalysed hydrolysis of methyl hippurate*

The hydrolyses were carried out at pH 6.0 in 0.3M-NaCl . Five points were used for each Lineweaver-Burk plot. For the catalytic constant, k_0 , $E_a = 11.6 \pm 1.5\text{kcal./mole}$, and $\Delta S^\ddagger = -26 \pm 5$ entropy units/mole at 35° .

Temp.	K_m (mM)	k_0 (sec. $^{-1}$)	k_0/K_m ($\text{M}^{-1}\text{sec.}^{-1}$)
25°	11.3	1.65	105
30	14.1	1.89	132
35	20.5	2.72	133
40	30.4	3.96	130

Table 3. *Papain-catalysed hydrolysis of hippuric esters*

The hydrolyses were carried out at 35° and pH 6.0 in 0.3M-NaCl . The numbers of points for the Lineweaver-Burk plots are given in parentheses; the numbers of Lineweaver-Burk plots are given in square brackets.

Ester (hippurate)	K_m (mM)	k_0 (sec. $^{-1}$)	k_0/K_m ($\text{M}^{-1}\text{sec.}^{-1}$)	[E] (μM)	[S] (mM)
Methyl	20.5 ± 2	2.72 ± 0.06	133 ± 3	1.02	5-30 (6)
Ethyl	27.0 ± 2	3.6 ± 0.3	133 ± 2	0.877	2-16 (6)
<i>n</i> -Propyl	18.2 ± 1	2.14 ± 0.09	118 ± 1	0.897	2-10 (5)
Isopropyl	45 ± 25	0.44 ± 0.24	9.8 ± 0.74	3.78	4-13 (5)*
<i>n</i> -Butyl	5.3 ± 0.5	1.29 ± 0.09	241 ± 2	3.03	3-20 (5)†
Thioethyl	16 ± 1	3.4 ± 0.2	206 ± 5	2.05	3-15 (4)†
2-Carboxythioethyl	5.2 ± 0.1	2.65 ± 0.05	502 ± 6	2.05	2-10 (5)‡
Phenyl	0.17 ± 0.015	3.02 ± 0.35	19000 ± 3200	1	0.09-0.3 (4)
<i>p</i> -Nitrophenyl	0.0140 ± 0.0034	2.31 ± 0.26	171000 ± 28000	1	0.007-0.04 [3]
<i>m</i> -Nitrophenyl	0.0183 ± 0.0006	2.47 ± 0.03	135000 ± 4200	1	0.03-0.18 [3]
<i>p</i> -Chlorophenyl	0.101 ± 0.023	3.96 ± 0.69	39500 ± 2800	1	0.02-1.8 [4]

* In aq. 12% (v/v) acetone.

† In aq. 20% (v/v) acetone.

‡ In aq. 8% (v/v) acetone.

The observed catalytic constant, k_0 , for hippurylamide (0.6 sec.^{-1}) (Smith, Chavré & Parker, 1958) and for *n*-butyl (1.3 sec.^{-1}) and isopropyl (0.4 sec.^{-1}) esters is, however, significantly lower than that for the other hippuric esters (see Table 3). To account for this difference it becomes necessary to postulate a three-step mechanism:

where ES is the Michaelis-Menten complex and ES' is the acyl-enzyme. Although the k_{-2} and k_{-3} steps can be neglected when initial velocities are taken ($[\text{P}_1]$ and $[\text{P}_2]$ being negligible), both the observed Michaelis-Menten parameters, k_0 and K_m , become composite:

$$k_0 = \frac{k_{+2}k_{+3}}{k_{+2} + k_{+3}} \quad \text{and} \quad K_m = \frac{k_{+3}(k_{-1} + k_{+2})}{k_{+1}(k_{+2} + k_{+3})}$$

The ability of papain to promote acyl transfer from a substrate to an α -amino acid ester (Sun Yu-Kun & Tsou Chen-Lu, 1963), aniline (Greenstein, 1954) or ammonia (Fruton, 1950) provides evidence that at least in amide hydrolysis (i.e. P_1 is an amine or ammonia) both k_{-1} and k_{-2} are significantly large. No evidence is provided in the literature to show whether esters can be synthesized by papain. We therefore allowed papain to hydrolyse a solution of methyl hippurate in water containing ^{14}C -methanol. When hydrolysis was 50% complete, the reaction was stopped and the solution worked up for methyl hippurate. The ester was shown to have incorporated only about 0.2% of the ^{14}C -methanol. This result is not surprising, since it is well established that thiol esters are much more susceptible to attack by nitrogen than oxygen nucleophiles (Bruice, 1961). For this reason it seems more likely that the ^{14}C -methyl hippurate is formed by methanolysis of the proposed reactive acyl-imidazole-enzyme rather than the relatively stable acylthiol-enzyme intermediate. The incorporation of 0.2% is therefore an upper limit to incorporation through the back-reaction (k_{-2} and k_{-1}).

To account for the essentially similar values of the observed catalytic constant, k_0 , for most hippuric esters, a three-step mechanism must be postulated in which $k_{+2} \gg k_{+3}$ and therefore $k_0 = k_{+3}$, the

deacylation constant. For the *n*-butyl and isopropyl esters and hippurylamide $k_{+2} \sim k_{+3}$ and therefore $k_0 < k_{+3}$ by the factor $k_{+2}/(k_{+2} + k_{+3})$, and it is possible to calculate the acylation constant, k_{+2} , and the enzyme-substrate equilibrium constant K_s (i.e. k_{-1}/k_{+1}) (see Table 4).

For those esters where $k_{+2} \gg k_{+3}$:

$$k_0/K_m = k_{+1}k_{+2}/(k_{-1} + k_{+2})$$

there are three possible relationships between k_{+2} and k_{-1} , namely:

$$k_{+2} \gg k_{-1} \quad \text{for which} \quad k_0/K_m = k_{+1}$$

$$k_{+2} \ll k_{-1} \quad \text{for which} \quad k_0/K_m = k_{+1}k_{+2}/k_{-1}$$

$$k_{+2} \sim k_{-1} \quad \text{for which} \quad k_0/K_m = k_{+1}k_{+2}/(k_{-1} + k_{+2})$$

The much greater values of k_0/K_m for the series of aryl esters of hippuric acid as compared with the

Fig. 1. Hammett plot for the papain-catalysed hydrolysis of some hippuric aryl esters: $\rho = 1.2$.

Table 4. Some kinetic constants for the papain-catalysed hydrolysis of some hippuric acid derivatives

k_{+3} is the catalytic constant for methyl hippurate. K_s was calculated assuming $k_{-1} \gg k_2$.

Substrate	k_0 (sec. ⁻¹)	k_{+3} (sec. ⁻¹)	k_{+2} (sec. ⁻¹)	K_s (mM)
Hippurylamide (Smith, Chavré & Parker, 1958)	0.6	2.7	0.77	202
Isopropyl hippurate	0.4	2.7	0.47	48
<i>n</i> -Butyl hippurate	1.3	2.7	2.47	10.2

alkyl esters make it extremely likely that this reflects the acylation step, k_{+2} , and therefore $k_{+2} \gg k_{-1}$ is not a solution of these kinetic data. The alternative solutions both involve the acylation step, k_{+2} , but, when $k_{+2} \ll k_{-1}$, k_0/K_m is linearly related to k_{+2} , whereas, when $k_{+2} \sim k_{-1}$, it is not. A distinction between those two situations should be possible by determining whether $\log(k_0/K_m)$ is linearly related to the Hammett substituent constants, since the latter should be linearly related to $\log k_{+2}$. The Michaelis-Menten parameters for the papain-catalysed hydrolysis of a series of aryl esters of hippuric acid are shown in Table 3. A plot of $\log(k_0/K_m)$ against σ for the aryl esters gives a good straight line with $\rho = 1.2$ (Fig. 1). The fact that a straight-line plot is obtained suggests that $k_{+2} \ll k_{-1}$, and further that the enzyme-substrate equilibrium constant, k_{-1}/k_{+1} , is essentially constant.

If we accept that the Hammett plot is a true reflexion of k_{+2} , the acylation step, then the fact that the substituent constant, σ , gives a better plot than σ^- , and that k_{+2} is relatively insensitive to change in substituent ($\rho = 1.2$) (preliminary unpublished results indicate that, for thiolate ion-catalysed hydrolysis of aryl acetates, $\rho = 1.7$), suggests that acylation, k_{+2} , is subject to acid as well as nucleo-

philic catalysis. This conclusion is supported by evidence in the literature that has not been interpreted. Stockell & Smith (1957) have shown that k_0/K_m for *N*- α -benzoyl-L-argininamide exhibits an inverse deuterium oxide solvent kinetic isotope effect. This unusual occurrence is nevertheless a characteristic of acid-catalysed ester hydrolysis (Bell, 1941). We therefore tentatively propose that the alkaline limb of the bell-shaped pH-rate profile for the acylation (more correctly, binding and acylation) step is due to the conjugate acid of an amino or an imidazole group. The kinetically determined pK (8.02 at 37°) and heat of ionization (5.1 kcal./mole at 0°) data favour the imidazolium ion [data from Dixon & Webb (1964): ϵ -amino group of lysine, pK 9.4–10.6 (25°), ΔH 10–12 kcal./mole; imidazole of histidine, pK 5.6–7.0 (25°), ΔH 6.9–7.5 kcal./mole]. Since papain contains only two histidine residues (Smith, Stockell & Kimmel, 1954), it seems probable that, if the above interpretation is correct, the same histidine residue is involved in both the acylation (as the imidazolium ion) and deacylation (as the free imidazole) steps.

Recent evidence by Sun & Tsou (1963) has shown that, for several *N*-acyl- α -amino acid amides, k_0 is bell-shaped and K_m is independent of pH. These observations are in complete accord with a three-

Scheme 1. Tentative mechanism of action for the papain-catalysed hydrolysis of *N*-acyl- α -L-amino acid derivatives.

step mechanism for papain-catalysed hydrolysis and allow us to derive the pH-dependencies of all three steps.

If $k_{+2} \gg k_{+3}$ and $k_{+2} \ll k_{-1}$, then $k_0 = k_{+3}$ and $k_0/K_m = k_{+1}k_{+2}/k_{-1}$

If, however, $k_{+3} \gg k_{+2}$, then $k_0 = k_{+2}$

and $K_m = k_{-1}/k_{+1}$

It therefore follows that the true acylation constant, k_{+2} , has a bell-shaped pH-dependence and that the enzyme-substrate equilibrium constant, k_{-1}/k_{+1} , and the true deacylation constant, k_{+3} , are pH-independent.

We can now summarize a new tentative mechanism for the papain-catalysed hydrolysis of *N*-acyl- α -L-amino acid derivatives (Scheme 1).

We thank the Department of Scientific and Industrial Research for a research studentship to A. W.

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