Direct Evidence for an Acylated Thiol as an Intermediate in Papain- and Ficin-Catalysed Hydrolyses

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1. Methyl thionhippurate was prepared and shown to be a specific substrate for both papain and ficin.
2. The ultraviolet-absorption properties of the acyl-enzyme intermediate for both papain and ficin with methyl thionhippurate was that expected of an acyl-thiol. The possibility that other functional groups present in papain or ficin might be the site of acylation has been excluded.
3. The change in extinction at the absorption maximum with time was as expected for the acyl-enzyme on the basis of the known Michaelis-Menten parameters for methyl thionhippurate.
4. The variation of extinction with initial substrate concentration for both papain and ficin was that expected from the Michaelis-Menten parameters.
5. The extinction of the absorption maximum of the thionhippuryl-enzyme intermediate was suppressed by the addition of methyl hippurate to the extent predicted from the Michaelis-Menten parameters.
6. The decay of the extinction for the acyl-enzyme was arrested by adjusting the pH of the solution to 2.5.
7. These experiments provide compelling evidence that the acylation by substrate of both papain and ficin takes place through a thiol residue.

The structure and mechanism of action of the crystalline proteolytic enzyme, papain, has been extensively investigated, especially by E. L. Smith and his collaborators, and has been reviewed on several occasions (Kimmel & Smith, 1957; Smith, Hill & Kimmel, 1958; Smith & Kimmel, 1960; Smith, Light & Kimmel, 1962). As a result of these researches, long sequences of amino acid residues have been established, and there is reason to hope that continuation of this work will lead to a complete elucidation of the primary structure of the enzyme. The tertiary structure is being investigated by Drenth and his collaborators (Drenth & Jansonius, 1959; Drenth *et al.* 1962) by X-ray analysis.

The hypothesis that papain depends for its enzymic activity on a thiol residue was based on the fact that oxidizing agents, e.g. hydrogen peroxide, iodine and oxygen, inactivate the enzyme, whereas reagents known to be capable of reducing disulphide bonds, e.g. hydrogen cyanide, hydrogen sulphide and thiols, reactivate the enzyme (Bersin, 1935). Mercuric salts and organomercurials also inactivate the enzyme (Kimmel & Smith, 1954; Finkle & Smith, 1958). More definitive evidence for an essential thiol residue was obtained when it was shown that the irreversible inhibition of papain by iodoacetamide at pH 8.2 was due to the carbamoylmethylation of a cysteine residue (Finkle & Smith, 1958).

The closely related crystalline proteolytic enzyme, ficin, is subject to activation and inactivation by reagents similar to those for papain (Bernhard & Gutfreund, 1956), and the irreversible inhibition of ficin by chloroacetamide and iodoacetamide has been shown to be due to the carbamoylmethylation of a group with pK 8.55, which was shown to be a cysteine residue (Hollaway, Mathias & Rabin, 1964).

Although such experiments suggest that enzymic activity depends on an essential thiol group, the evidence is equivocal since such inhibitors may simply modify the tertiary structure of the active centre without necessarily reacting with it. Attention has been drawn on several occasions to the dangers inherent in drawing firm conclusions from the results of such inhibition experiments.

The kinetics of hydrolysis of *N*-acyl- α -amino acid esters and amides by papain and ficin can be analysed in terms of the Michaelis-Menten equation. For each enzyme the observed catalytic rate constants, k_0 [from the breakdown of the initial rate, $v = k_0[E][S_0]/\{K_m + [S_0]\}$], for *N*- α -benzoyl-L-arginine ethyl ester and *N*- α -benzoyl-L-argininamide were the same (Smith & Parker, 1958; Stockell & Smith, 1957; Hammond & Gutfreund, 1959), and it was argued that the observed catalytic step involved the deacylation of the common acyl-enzyme intermediate; e.g.

It was further argued that $k_{-1} \ll k_0$ and therefore that $k_0/K_m = k_{+1}$, the acylation step. The pH- k_{+1} profile was bell-shaped with half-maximal values at pH 4.30 and 8.02 for papain (Smith & Parker, 1958); for ficin the pH-rate profile was bell-shaped with half-maximal rates at pH 4.40 and 8.46 (Hammond & Gutfreund, 1959). It was suggested that the acid limb represented a carboxylic acid group in both papain and ficin. The alkaline limb in papain-catalysed hydrolyses was interpreted as being due to the ionization of the essential thiol residue, whereas in ficin it was regarded as being due to an amino group. Acylation of each enzyme was, however, thought to occur through an essential thiol group.

If it is accepted that an acyl-enzyme is formed, and there is now powerful evidence for such a hypothesis for both enzymes (Smith & Parker, 1958; Hammond & Gutfreund, 1959; Lowe & Williams, 1965), then the keystone to the mechanisms of action must be the nature of the acyl-enzyme bond. The available evidence that the site of acylation is a thiol group is not compelling.

If the kinetically observed catalytic step, k_0 , in papain- and ficin-catalysed hydrolyses is the deacylation of the acyl-enzyme intermediate, there must be an appreciable steady-state concentration of such an intermediate. Therefore, by using a substrate that will give an acyl-enzyme intermediate with a characteristic ultraviolet absorption distinguishable from the absorption bands of the enzyme, it should be possible to detect it spectroscopically. If an acylated thiol is the intermediate in papain- or ficin-catalysed hydrolyses of *N*-acyl- α -amino acid esters or amides, then by using a thiono ester (e.g. I) as a substrate it should be possible to measure the steady-state concentration of the dithio ester intermediate (II) directly, since its expected intense absorption band near 3050 Å is at a longer wavelength than the absorption bands of the enzyme itself (Glazer & Smith, 1961) [e.g. $\text{CH}_3 \cdot \text{CS}_2 \cdot \text{C}_2\text{H}_5$, λ_{max} (in heptane or water) 3050 Å ($\log \epsilon$ 4.08) and 4600 Å ($\log \epsilon$ 1.25) (Rao, 1961; G. Lowe & A. Williams, unpublished work)].

The thiono ester (I) was prepared from the nitrile (III) (Klages & Haack, 1903) by treatment first with methanolic hydrogen chloride to give the imino ether hydrochloride (IV), which with pyridine

and hydrogen sulphide gave methyl thionohippurate (I).

The work described in the present paper has been the subject of a preliminary communication (Lowe & Williams, 1964).

MATERIALS AND METHODS

Papain. Twice-recrystallized papain (British Drug Houses Ltd., Poole, Dorset) was activated with *p*-thiocresol (Soejima & Shimura, 1961). The concentration of *p*-thiocresol in the final preparation was estimated to be less than 10 μM . The enzyme concentration was estimated by using *N*- α -benzoyl-L-arginine ethyl ester as substrate and the data given by Smith & Parker (1958).

Ficin. Twice-recrystallized ficin (L. Light and Co. Ltd., Colnbrook, Bucks.) was activated as above. The enzyme concentration was estimated by using methyl hippurate as substrate and the data given by Hammond & Gutfreund (1959).

Methyl hippurate. This was prepared as described by Hammond & Gutfreund (1959) and had m.p. 80–81.5°.

Methyl thionohippurate. A solution of hippuronitrile (8.0 g.) (Klages & Haack, 1903) in chloroform (80 ml.) and benzene (80 ml.) containing methanol (2 ml.) was treated with dry HCl for 30 min. On cooling to 0° *O*-methyl imidohippurate hydrochloride (11.0 g.) crystallized out, m.p. 127° (Found: C, 52.2; H, 6.1; Cl, 15.7; N, 12.1. $\text{C}_{10}\text{H}_{13}\text{ClN}_2\text{O}_2$ requires C, 52.5; H, 5.7; Cl, 15.5; N, 12.2%).

A solution of the imino ether hydrochloride (0.8 g.) in pyridine (1 ml.) and methanol (5 ml.) was treated with H_2S for 15 min. at 20° and the solution kept for another 30 min. Ammonium chloride was precipitated, and on the addition of 2*N*-HCl crystals of methyl thionohippurate (0.5 g.) were obtained. Recrystallization from acetone-water gave needles, m.p. 83–84° (Found: C, 57.4; H, 5.2; N, 6.6; S, 15.5. $\text{C}_{10}\text{H}_{11}\text{NO}_2\text{S}$ requires C, 57.5; H, 5.3; N, 6.7; S, 15.3%), $\lambda_{\text{max}}^{\text{EtOH}}$ 2300 Å (ϵ 18000), ν_{max} (Nujol) 3300 (NH) and 1640 cm^{-1} (CO-NH).

Kinetics. (a) Titrimetric method. The Michaelis-Menten parameters for papain- and ficin-catalysed hydrolyses were determined in the thermostatically controlled cell of a pH-stat [Radiometer titrator (type TTT1c), titratigraph recorder (type SBR2c) and a syringe burette (type SBU1a), which delivered 0.5 ml. of base at full-scale deflexion of the recorder]. The reaction rate was determined from the recorded plot of 0.02*N*-NaOH solution added, to maintain the solution at pH 6.0. By determining the initial velocities, v (less than 5% of total substrate had reacted), at different substrate concentrations the observed Michaelis-Menten parameters, k_0 and K_m , were obtained from:

$$v = k_0[\text{E}][\text{S}_0]/(K_m + [\text{S}_0])$$

by using a Lineweaver-Burk plot and the method of least

squares (an Autocode computer programme employing a Ferranti Mercury digital computer enabled the least-squares results to be calculated automatically from the kinetic data). The results are shown in Table 1.

(b) Spectrophotometric method. Four 1 cm. quartz cells were placed in the cell compartment of a Unicam SP.800 spectrometer. Into one of the cells in the 'compensating beam' was placed the enzyme solution, and into the other was placed a solution of methyl thionohippurate in phosphate buffer, pH 6.0. Into one of the cells in the 'solution beam' was placed phosphate buffer solution and into the other (i.e. the reaction cell) enzyme solution. When the solutions had reached thermal equilibrium (approx. 20°) methyl thionohippurate in acetone-water (1:62.5, v/v) was added to the reaction cell and the solution thoroughly mixed.

RESULTS

The observed Michaelis-Menten parameters, k_0 and K_m , for the papain- and ficin-catalysed hydro-

lyses of methyl thionohippurate were determined by pH-stat and are shown in Table 1. The Michaelis-Menten parameters for methyl hippurate are shown in Table 2 for comparison. For solubility reasons, the methyl thionohippurate parameters were determined in aqueous acetone.

When the papain- and ficin-catalysed hydrolyses of methyl thionohippurate are followed spectrophotometrically a transient absorption band appears at 3130 Å with papain and at 3150 Å with ficin. The change in the extinction of the absorption bands with time is shown in Tables 3 and 4 respectively.

Table 5 shows the effect on the extinction (measured about 30 sec. after mixing) at 3130 and 3150 Å for papain- and ficin-catalysed hydrolyses respectively with different initial methyl thionohippurate concentrations and constant enzyme concentrations. Table 6 shows the suppression of the

Table 1. *Papain- and ficin-catalysed hydrolyses of methyl thionohippurate*

The hydrolyses were carried out at 35° and pH 6.0 in 0.3M-NaCl in aq. 30% (v/v) acetone. The numbers of determinations at different concentrations for each Lineweaver-Burk plot are given in parentheses.

Enzyme	[E] (μ M)	[S ₀] (mM)	k_0 (sec. ⁻¹)	K_m (mM)	k_0/K_m (M ⁻¹ sec. ⁻¹)
Papain	3.0	3-25 (7)	0.14	6.3	22.8
Ficin	9.5	2-12 (5)	0.06	7.7	7.7

Table 2. *Papain- and ficin-catalysed hydrolyses of methyl hippurate*

The papain-catalysed hydrolyses were carried out at 35° and pH 6.0 in (a) 0.3M-NaCl and (b) 0.3M-NaCl in aq. 30% (v/v) acetone. The ficin-catalysed hydrolyses were carried out at 25° and pH 6.06 in M-NaCl by Hammond & Gutfreund (a mol. wt. 25000 was assumed for ficin).

Enzyme	[E] (μ M)	[S ₀] (mM)	k_0 (sec. ⁻¹)	K_m (mM)	k_0/K_m (M ⁻¹ sec. ⁻¹)
Papain (a)	1	5-30	2.7	20.5	133
(b)	7.7	16-60	1.5	73	20.5
Ficin	~200	2.5-32.5	3.5	48.0	73

Table 3. *Variation with time of the extinction at the absorption maximum (3130 Å) for the papain-catalysed hydrolysis of methyl thionohippurate (I)*

Papain [69 μ M, in phosphate buffer, pH 6.0, containing NaCl (0.3M)] was mixed with methyl thionohippurate [2.53 mM, in acetone-water (1:62.5, v/v)] and the hydrolyses were carried out at approx. 20°.

Time (min.)	0.5	4.0	5.5	7.0	9.0	12.5
$E_{3130 \text{ Å}}$	0.26	0.23	0.18	0.12	0.04	0

Table 4. *Variation with time of the extinction at the absorption maximum (3150 Å) for the ficin-catalysed hydrolysis of methyl thionohippurate (I)*

Ficin [3.4 mM, in phosphate buffer, pH 6.0, containing NaCl (0.3M)] was mixed with methyl thionohippurate [3.62 mM, in acetone-water (1:62.5, v/v)] and the hydrolyses were carried out at approx. 20°.

Time (min.)	1	4	17	26	29	32	35
$E_{3130 \text{ Å}}$	0.38	0.40	0.41	0.31	0.28	0.24	0.23

extinction (measured about 30sec. after mixing) of the transient absorption bands in the presence of methyl hippurate.

When a solution of methyl thionohippurate and papain or ficin was adjusted to pH 2.5 about 30sec. after mixing, the extinction of the absorption maximum (shifted to 3090 and to 3130Å respectively) was maintained.

DISCUSSION

The Michaelis-Menten parameters for the papain- and ficin-catalysed hydrolyses of methyl thiono-

Table 5. *Steady-state extinction measured for different initial concentrations of methyl thionohippurate*

The hydrolyses were carried out at approx. 20° at pH 6.0 in 0.3M-NaCl. The extinctions were measured at 3130Å (for papain) or at 3150 Å (for ficin) 30sec. after the substrate and enzyme solutions had been mixed.

Enzyme	[E] (μM)	[S ₀] (mM)	<i>E</i> _{max.}	<i>E</i> _{calc.}
Papain	69	0.55	0.065	0.053
	69	1.10	0.108	0.098
	69	2.20	0.170	0.170*
Ficin	190	0.643	0.065	0.055
	190	1.93	0.140	0.140
	190	3.21	0.210	0.210*

* Observed value assumed to be accurate, the other values being calculated from this by using *K_m* for methyl thionohippurate at 35° in acetone-water (3:7, v/v).

hippurate were determined by pH-stat at pH 6.0 and 35° (see Table 1). For solubility reasons, however, it was necessary to use aqueous acetone as solvent. The Michaelis-Menten parameters for the hydrolysis of methyl hippurate by these enzymes in water, and by papain in aqueous acetone, under the same conditions are shown in Table 2. As expected, the addition of an organic solvent does influence the Michaelis-Menten parameters, but not to an extent that a change of mechanism is likely. A comparison of the Michaelis-Menten parameters for the papain-catalysed hydrolysis of methyl hippurate and methyl thionohippurate in aqueous acetone reveals that the observed catalytic constants, *k*₀, differ by a factor 10 [it is shown by Lowe & Williams (1965) that *k*₀ represents deacylation of the acyl-enzyme, and therefore *k*₀/*K_m* represents binding and acylation], whereas the binding and acylation steps, *k*₀/*K_m*, are comparable. [The OH⁻-catalysed hydrolysis of thiono esters is virtually identical with that of oxygen esters (Smith & O'Leary, 1963).] There can be little doubt therefore, from these data, that methyl thionohippurate is a specific substrate for these enzymes, but further evidence to support this view is referred to below.

From the Michaelis-Menten parameters for the enzyme-catalysed hydrolysis of methyl thionohippurate it was possible to calculate the steady-state concentration of the acyl-enzyme intermediate, assuming that *k*_{acylation} ≫ *k*_{deacylation}. From the known ultraviolet-absorption properties of dithio esters an experiment was designed that would enable the

Table 6. *Suppression of the steady-state extinction by the addition of methyl hippurate*

The hydrolyses were carried out at approx. 20° at pH 6.0 in 0.3M-NaCl. The extinctions were measured at 3130 Å (for papain) or at 3150 Å (for ficin). *E*₁ is the steady-state extinction in the absence of methyl hippurate at the absorption maximum. *E*₂ is the steady-state extinction in the presence of 33.4mM-methyl hippurate (for papain) or 35.3mM-methyl hippurate (for ficin) at the absorption maximum, 30sec. after the substrate and enzyme solutions had been mixed. *E*₁/*E*₂ (calc.) was calculated by using *K_m* for methyl hippurate and methyl thionohippurate at 35° and in aq. 30% (v/v) acetone.

Enzyme	[E] (μM)	[S ₀] (mM)	<i>E</i> ₁	<i>E</i> ₂	<i>E</i> ₁ / <i>E</i> ₂	<i>E</i> ₁ / <i>E</i> ₂ (calc.)
Papain	69	4.1	0.523	0.276	1.9	2.0
Ficin	206	3.62	0.38	0.30	1.3	1.5

Table 7. *Ultraviolet absorption properties of thiono-acyl derivatives*

Chromophore	Solvent	λ _{max.} (Å)	log ε	λ _{max.} (Å)	log ε	Reference
-CS·OR	Ethanol	2300	4.26	—	—	This paper
-CO·S ⁻	Water	2490	2.75	—	—	Wieland & Bartmann (1956)
-CS·NH ₂	Ether	2680	4.05	3580	1.25	Rao (1961)
-CS·N	—	2500	4.00	2910	4.00	H. A. Staab (personal communication)
-CS·SR	Hexane*	3050	4.08	4600	1.25	Rao (1961)
Papain-CS·R	Water	3130	4.2	—	—	
Ficin-CS·R	Water	3150	3.7	—	—	

*Ethyl dithioacetate has λ_{max.} 3050 Å in water (G. Lowe & A. Williams, unpublished work).

detection of the acyl-enzyme intermediate, if this involved a dithio ester linkage. The ultraviolet-absorption data in Table 7 indicate that an absorption band near to 3050 Å would provide unequivocal evidence for the acyl-enzyme being formed through a thiol residue.

In the event, when the ultraviolet-absorption spectrum (3600–2000 Å) of a solution of enzyme and methyl thionohippurate was measured, with separate solutions of enzyme and methyl thionohippurate of the same concentration in the compensating beam of the spectrometer, an absorption band with maximal absorption at 3130 and 3150 Å for papain and ficin respectively was observed 30 sec. after mixing. The steady-state extinctions (Table 5) of this peak for different initial substrate concentration were in reasonably good agreement with the prediction from the known Michaelis–Menten parameters (Table 1), considering that they were determined under somewhat different conditions. The change in extinction of this peak with time (Tables 3 and 4) is that expected of an acyl-enzyme intermediate as the substrate is consumed. The $\log \epsilon$ was calculated for thionohippuryl-papain and -ficin as approx. 4.2 and 3.7 respectively.

The next experiment was designed to reveal competition between methyl hippurate and methyl thionohippurate for the enzyme. If methyl hippurate suppressed the absorption band with methyl thionohippurate in a way predictable from the Michaelis–Menten parameters for an acyl-enzyme intermediate, then it is reasonable to assume that these substrates are reacting at the same site on the enzyme. The results in Table 6 show the extent to which the extinction is suppressed; they agree well with prediction, probably owing partly to the cancelling effects of change in solvent and temperature.

Finally, when a solution of methyl thionohippurate and the enzyme was adjusted to pH 2.5 the decay of the absorption maximum (shifted to 3090 and 3130 Å for papain and ficin respectively) was arrested. The enzymes are largely denatured at this pH, thus preventing deacylation of the acyl-enzyme.

These experiments support the view that the absorption band generated between methyl thiono-

hippurate and the enzymes, papain and ficin, is due to the acyl-enzyme intermediate. The wavelength of the absorption maximum and its extinction coefficient provide direct evidence that papain and ficin are acylated through a thiol residue.

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