A Study of some Thiol Ester Hydrolyses as Models for the Deacylation Step of Papain-Catalysed Hydrolyses

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1. The self-catalysed hydrolyses of the thiol esters, *S*-hippurylthioglycollic acid and *S*-ethyl monothiolsuccinate, have been shown to be slower than the deacylation step for the papain-catalysed hydrolysis of hippuric esters, by a factor approx. 10^5 . This difference in rate constants largely reflects a difference in activation energy, which together with other evidence drawn from the literature make it unlikely that a carboxylate ion could be the nucleophile responsible for the deacylation of acyl-papain. 2. The imidazole-catalysed hydrolysis of *S*-hippurylthioglycollic acid and ethyl thiolacetate have activation energies similar to that for the deacylation step in papain-catalysed hydrolyses. This, together with other evidence drawn from the literature, suggests that the imidazole of a histidine residue is the nucleophile responsible for the deacylation of acyl-papain.

A mechanism of action for papain has been proposed by Smith and his collaborators from a study of the kinetics of hydrolysis of a number of synthetic substrates, and has been reviewed on several occasions (Kimmel & Smith, 1957; Smith, Hill & Kimmel, 1958; Smith & Kimmel, 1960; Smith, Light & Kimmel, 1962). When the kinetics of the papain-catalysed hydrolysis of *N*- α -benzoyl-L-argininamide (Stockell & Smith, 1957) and *N*- α -benzoyl-L-arginine ethylester (Smith & Parker, 1958) were analysed in terms of the Michaelis-Menten equation, their observed catalytic rate constants, k_0 , were found to be similar, and the suggestion was made that this constant represents the deacylation of the common acyl-enzyme. The pH-dependence of the Michaelis-Menten parameters for *N*- α -benzoyl-L-arginine ethyl ester indicated that a group with apparent pK_a 3.5 at 25° played an important role in the deacylation of the enzyme (Smith & Parker, 1958) and the conclusion was drawn that deacylation involved a carboxylate ion-catalysed hydrolysis of the acyl-enzyme intermediate (Smith, 1958). Sluyterman (1964) has claimed that this conclusion was unjustified since a pH-stat was employed for following the kinetics and no correction was made for the state of ionization of the carboxyl group of the product, *N*- α -benzoyl-L-arginine. However, in a more recent study in which the papain-catalysed hydrolysis of *N*- α -benzoyl-L-argininamide and *N*- α -benzoyl-L-arginine ethyl ester was followed spectroscopically (Whitaker & Bender, 1965) (a correction was made for product ionization and inhibition), it was found

that the pH-deacylation profile was a sigmoid curve with pK 3.90. Sluyterman (1964) has found that deacylation of hippuryl-papain is pH-independent down to pH 4.2, and this has been confirmed in our Laboratory down to pH 3.8 (Williams, 1964). We therefore suggest that the pH-dependence of the deacylation of *N*- α -benzoyl-L-arginyl-papain is due to binding of the guanidino group by a carboxylate ion, thereby assisting deacylation by specifically orienting the thiol ester bond. This is borne out by the fact that at pH 3.0, when the carboxyl group is largely undissociated, the rate of deacylation ($k_{25^\circ} = 2.5 \text{ sec}^{-1}$) (Whitaker & Bender, 1965) is almost identical with that of hippuryl-papain ($k_{35^\circ} = 2.7 \text{ sec}^{-1}$) (Lowe & Williams, 1965b). Evidence is now presented from model studies which indicates that the group responsible for deacylation of acyl-papains is probably an imidazole residue of histidine.

MATERIALS AND METHODS

S-Hippurylthioglycollic acid (III). This was prepared by the method of Schwyzer (1958) and recrystallized from water as prisms, m.p. 142° [Schwyzer (1958) gives m.p. 142°] (Found: C, 52.3; H, 4.3; N, 5.3; S, 12.2%; equiv. wt. 253. Calc. for $C_{11}H_{11}NO_4S$: C, 52.2; H, 4.4; N, 5.5; S, 12.7%; equiv. wt. 253).

S-Ethyl monothiolsuccinate (IV) (cf. Mitsuo Masumura & Tokunaru Horie, 1959). Succinic anhydride (5.0 g.) was added to a stirred solution of ethanethiol (3.7 g.) in aq. 10% (w/v) NaOH solution (20 ml.) at 0° and after 30 min. the solution was extracted with ether. The aqueous solution was acidified and the crude product isolated with ether

was fractionally distilled. The *thiol ester* was a colourless liquid, b.p. 125–130°/0.55 mm. Hg, n_D^{20} 1.493 (Found: C, 44.6; H, 6.5. $C_6H_{10}O_3S$ requires C, 44.4; H, 6.2%), λ_{max} 2330 Å (ϵ 7700).

Ethylthiolacetate (V) (cf. Houben-Weil, 1955). Ethanethiol (8.9 ml.) was added to a solution of NaOH (4.0 g.) in water (7 ml.) and crushed ice (50 g.). The mixture was stirred at 0° while acetic anhydride (11.6 ml.) was added over 5 min. The product was extracted with ether, dried (over Na_2SO_4) and distilled, b.p. 115°/760 mm. Hg, n_D^{20} 1.450 [Bender (1957) (cf. also Rylander & Tarbell, 1950) gives b.p. 115°, n_D^{20} 1.458], λ_{max} 2300 Å (ϵ 3800), ν_{max} 1700 cm^{-1} (Found: C, 46.1; H, 7.8. Calc. for C_4H_8OS : C, 46.1; H, 7.7%).

Kinetics. (a) Titrimetric method. The titration cell contained 10 ml. of 0.1 M-NaCl thermostatically controlled by circulating water to $\pm 0.1^\circ$ and stirred magnetically. The pH of the solution was adjusted and maintained by a pH-stat apparatus [Radiometer titrator (type TTT1c), titrator recorder (type SBR2c) and a syringe burette (type SBU1a), which delivered 0.5 ml. of base at full-scale deflexion of the recorder]. Ester was added and the 0.02 N-NaOH solution required to maintain a constant pH recorded against time. Reactions were followed to at least 3 half-lives and the rate constants determined by the method of Guggenheim (1926).

(b) Spectrophotometric method. The disappearance of the thiol ester was followed in a modified Unicam SP.500 spectrophotometer fitted with a photomultiplier, the output being fed to a potentiometric pen recorder so that the transmission at a fixed wavelength was recorded against time. Reactions were followed to at least 3 half-lives and the rate constants determined by the method of Guggenheim (1926). The temperature of the cells was controlled to $\pm 0.1^\circ$ with an Adkins thermostat. From the known pH-rate profile for the hydrolysis of *N*-acetylimidazole (Jencks & Carriuolo, 1959), it was assumed that in these experiments the concentration of *N*-acyl-imidazole was negligible, its rate of hydrolysis being greater than its rate of formation.

RESULTS

Hydrolysis of *S*-hippurylthioglycollic acid (III). The rate of hydrolysis of *S*-hippurylthioglycollic acid was determined at a concentration of 1 mM in 0.1 M-sodium chloride solution by a pH-stat. The rate was essentially independent of pH between pH 4 and 7.7 (Table 1), from which the intramolecularly catalysed rate constant $k_{30^\circ} = 0.88 (\pm 0.16) \times$

$10^{-5} sec^{-1}$ was obtained. At pH values higher than 7.7 the rate increased due to OH^- catalysis to $k_{30^\circ} = 1.51 (\pm 0.17) M^{-1} sec^{-1}$. The rate of hydrolysis (sec^{-1}) was determined at pH 7.7 at 20° (0.43×10^{-5}), 25° (0.80×10^{-5}), 30° (1.16×10^{-5}), 35° (1.81×10^{-5}) and 40° (3.22×10^{-5}), from which the Arrhenius parameters were determined (Table 3) by the method of least squares.

Hydrolysis of *S*-ethylmonothiol succinate (IV). The rate of hydrolysis of *S*-ethyl monothiol succinate was determined at a concentration of 1 mM in 0.1 M-sodium chloride solution by a pH-stat. The rate was essentially independent of pH between pH 5 and 8 (Table 2), from which the intramolecularly catalysed rate constant $k_{30^\circ} = 3.2 (\pm 0.5) \times 10^{-5} sec^{-1}$ was determined. The rate of hydrolysis (sec^{-1}) was also determined at pH 7.2 at 25° (1.88×10^{-5}), 30° (3.51×10^{-5}), 35° (5.58×10^{-5}) and 40° (10.5×10^{-5}), from which the Arrhenius parameters were obtained (Table 3) by the method of least squares.

Table 1. pH-dependence of the hydrolysis of *S*-hippurylthioglycollic acid (III) at 30°

These data fit the equation $k = k_i + k_{OH^-}[OH^-]$, where $k_i = 0.88 (\pm 0.16) \times 10^{-5} sec^{-1}$ and $k_{OH^-} = 1.51 (\pm 0.17) M^{-1} sec^{-1}$.

pH	4.30	4.5	4.55	4.80	5.49	6.00	6.28	6.78
$10^5 k$	0.75	0.68	0.75	0.74	0.87	0.77	0.76	1.06
(sec. ⁻¹)								
pH	7.00	7.20	7.50	7.7	9.22	9.5	9.78	10.0
$10^5 k$	0.78	1.07	1.14	1.13	2.15	4.22	5.57	8.55
(sec. ⁻¹)								

Table 2. pH-dependence of the hydrolysis of *S*-ethyl monothiol succinate (IV) at 30°

$$k_i = 3.2 \times 10^{-5} sec^{-1}.$$

pH	4.90	5.00	6.10	6.69	7.20	7.70
$10^5 k$	3.0	2.8	3.9	3.5	3.1	3.1
(sec. ⁻¹)						

Table 3. Rate constants and Arrhenius parameters for some carboxylate ion-catalysed thiol ester hydrolyses compared with those for deacylation of some acyl-papains

Acyl-enzyme or model	Temp.	$10^5 k_i$ (sec. ⁻¹)	k_0 (sec. ⁻¹)	E_a (kcal./mole)	$\Delta S^\ddagger$ (entropy units/mole)	Reference
Benzoylarginyl-papain	38°	—	11	9.5	-23	Stockell & Smith (1957)
Hippuryl-papain	35	—	2.7	11.6 ± 1.5	-26 ± 5	Lowe & Williams (1965b)
(I)	25	0.7	—	—	—	Schonbaum & Bender (1960)
(II)	~25	10–20	—	—	—	Simon & Shemin (1953)
(III)	30	0.88	—	18.4 ± 1.8	-6 ± 6	This paper
(IV)	30	3.25	—	22.1 ± 1.3	$+8 \pm 4$	This paper

Table 4. Rate constants and Arrhenius parameters for imidazole-catalysed thiol ester hydrolyses compared with those for deacylation of some acyl-papains

Acyl-enzyme or model	Temp.	k (sec. ⁻¹)	$10^4 k$ (M ⁻¹ sec. ⁻¹)	E_a (kcal./mole)	$\Delta S^\ddagger$ (entropy units/mole)	Reference
Benzoylarginyl-papain	38°	11	—	9.5	-23	Stockell & Smith (1957)
Hippuryl-papain	35	2.7	—	11.6 ± 1.5	-26 ± 5	Lowe & Williams (1965b)
Toluene- <i>p</i> -sulphonyl- glycyl-papain (VI)	35	0.64	—	—	—	Williams (1964)
(III) + imidazole	15	0.089	—	—	—	Bruice (1959)
(V) + imidazole	38	—	45	9.9 ± 0.4	-38 ± 1.2	This paper
	35	—	6.2	7.9 ± 0.6	-50 ± 2	This paper

Imidazole-catalysed hydrolysis of S-hippurylthioglycollic acid (III). This part of the investigation was carried out in collaboration with Mr J. Leyshon. The hydrolysis of *S*-hippurylthioglycollic acid was followed spectroscopically at 2400 Å in phosphate buffer, pH 6.84, in the presence and absence of 0.05M-imidazole. The ester was added in aq. 10% (v/v) ethanol (0.05M) to give an initial concentration of 0.1mM. The imidazole-catalysed pseudo-first-order rate constants were corrected for self hydrolysis and the second-order rate constant in Table 4 was derived from the least-squares Arrhenius parameters assuming imidazole to have pK_a 6.9. The rate of hydrolysis (M⁻¹sec.⁻¹) was determined at 31.5° (3.21×10^{-3}), 38.0° (4.44×10^{-3}), 41.2° (5.20×10^{-3}), 45.4° (6.00×10^{-3}) and 50.4° (8.08×10^{-3}).

Imidazole-catalysed hydrolysis of ethyl thiolacetate (V). The hydrolysis of ethyl thiolacetate was followed spectroscopically at 2300 Å. A 2.5ml. portion of aqueous imidazole solution (0.741M, brought to pH 8.3 with concentrated hydrochloric acid) was equilibrated in the thermostatically controlled cell compartment of the spectrometer, and 5 μl. of a solution of ethyl thiolacetate (0.05M, in acetonitrile) added. The pseudo-first-order rate constants were obtained without correction for OH⁻ catalysis (Schaefer, 1948). The second-order rate constant in Table 4 was derived from the least-squares Arrhenius parameters, assuming imidazole to be completely in the basic form. The rate of hydrolysis (M⁻¹sec.⁻¹) was determined at 35° (8.18×10^{-5}), 40° (9.31×10^{-4}), 45° (12.2×10^{-4}) and 50° (14.9×10^{-4}).

DISCUSSION

The literature provides only two examples of intramolecularly catalysed hydrolyses of thiol esters by the carboxylate ion. From the pH-rate profile, the intramolecularly catalysed hydrolysis of thioaspirin (I) had k_{25} $7.0 (\pm 3.0) \times 10^{-6}$ sec.⁻¹ (Schonbaum & Bender, 1960). The intramolecularly catalysed hydrolysis of *S*-succinyl-CoA (II) was

said to have a half-life of 1–2 hr. at pH 7 and room temperature (Simon & Shemin, 1953), from which a very approximate rate constant of 1×10^{-4} – 2×10^{-4} sec.⁻¹ could be calculated. Arrhenius parameters, however, were not determined in either case.

We chose as a model compound *S*-hippurylthioglycollic acid (III), since the rate constant for the deacylation of hippuryl-papain is known (Lowe & Williams, 1965b). The acyl residue of the substrate is also known to be bonded to the enzyme in the acyl-enzyme through a thiol residue (Lowe & Williams, 1964, 1965a; Bender & Brubacher, 1964), and the carboxylate ion is a possible nucleophile for the deacylation process. The carboxylic acid group in the model is favourably situated for intramolecular reaction with the thiol ester group. A second model that was investigated was *S*-ethyl monothiol succinate, since in this case the carboxylic acid and the thiol ester are oriented differently towards each other.

The rate constants for hydrolysis of the thiol esters (III) and (IV) were measured at different pH values (Tables 1 and 2). The intramolecularly catalysed hydrolysis constants were obtained from the region in which the rate was pH-independent. As shown in Table 3, the four model systems (I, II, III and IV) have comparable rate constants but differ by a factor approx. 10^5 from the deacylation rate constant for the papain-catalysed hydrolysis of methyl hippurate. It was decided, however, to investigate whether this difference arose from an unfavourable entropy or energy of activation. The influence of temperature on the rate constant for the hydrolysis of the thiol esters (III) and (IV) was therefore measured at pH 7.7 and 7.2 respectively, where the carboxylic acid groups were fully ionized and OH⁻-catalysed hydrolysis was negligible. From these results the Arrhenius parameters were determined (Table 3). For both model compounds (III) and (IV) the entropy of activation is more favourable than that of the enzyme-catalysed reaction (Table 3). It could not therefore be reasonably argued that the difference in rate

(I)

(II)

(III)

(IV)

(V)

(VI)

Scheme 1.

constants was due to the more favourable arrangement on the enzyme surface. The essential difference between the rate of hydrolysis of the acyl-enzyme and the model is mainly a reflexion of a difference in activation energies. This observation suggests that a nucleophile other than the carboxylate ion is involved in the enzymic deacylation process. The possibility of concerted nucleophilic attack (by a carboxylate ion) and general acid catalysis cannot be rigorously excluded, but from the evidence that follows this seems less likely.

It is an established fact that nitrogen nucleophiles are very much more reactive than oxygen nucleophiles towards thiol esters (Bruice, 1961); further, imidazole acting as a nucleophile would lead to a labile intermediate. Only one example of the hydrolysis of a thiol ester catalysed intramolecularly by imidazole has been reported. *n*-Propyl α -(4-imidazolyl)thiobutyrate (V) was shown to have an intramolecular-imidazole-catalysed rate constant 0.089 sec^{-1} at 30° (Table 4) (Bruice, 1959). Although this is still somewhat lower than the enzymic deacylation constant for *N*- α -benzoyl-L-arginine ethyl ester and methyl hippurate it is in reasonably good agreement with some other substrates, e.g. *N*-toluene-*p*-sulphonylglycine methyl ester (Table 4) (Williams, 1964). Unfortunately, no Arrhenius parameters were determined for this reaction. The intermolecularly imidazole-catalysed hydrolyses of *S*-hippurylthioglycolic acid (III) and ethyl thiolacetate (V) have been studied therefore and the Arrhenius parameters determined. Since

the reactions were bimolecular only a comparison of the activation energies with that for the enzyme was justifiable. The rate constant and entropy of activation are, as expected, very different from that of the deacylation constants for benzoylarginyl-papain and hippuryl-papain, but the activation energy is remarkably similar (Table 4), thus supporting the view that imidazole may be the nucleophile involved.

It has been shown that the site of attachment of the carboxymethyl group in papain, irreversibly inhibited with bromoacetic acid at pH 5.0, is probably the imidazole group of a histidine residue (Sun Yu-kun & Tsou Chen-Lu, 1963b; Lu-Hsiu Pan & Yu-Kun Sun, 1963). Doubt has been cast on this observation by Light (1964), who repeated the experiment and found that only a cysteine residue was carboxymethylated. This constitutes the first piece of direct experimental evidence implicating a histidine residue in the active site of papain and is further supported by photo-oxidation experiments (Sun-Yu-Kun & Tsou Chen-Lu, 1963a). It seems probable that this same imidazole residue is the nucleophile responsible for the deacylation step in papain-catalysed hydrolyses (Scheme 1).

There remains to be considered the pH-independence of the deacylation reaction, since the imidazole group would normally be expected to have pK_a in the range 5.6–7.0 (Dixon & Webb, 1958). We favour the view that the imidazole residue in the acyl-enzyme is 'buried', thus making it inaccessible to protons. Imidazole residues are known to be

in a similar situation in sperm-whale myoglobin (Breslow & Gurd, 1962; Steinhardt, Ona & Beychock, 1962).

From the known rate of hydrolysis of acetyl-imidazole in water (k_{21} , $2.9 \times 10^{-4} \text{sec.}^{-1}$) (Staab, 1959) it seems unlikely that the *N*-acyl-enzyme would break down spontaneously at a sufficiently fast rate to be kinetically unobserved. However, it is known that the hydrolysis of the acetyl-imidazolium ion in water is considerably faster (k_{25} , 0.048sec.^{-1}) (Jencks & Carriuolo, 1959) and, moreover, is subject to general base catalysis (Wolfenden & Jencks, 1961). We therefore suggest that the breakdown of the *N*-acyl-enzyme is not a kinetically observable step because the acyl-imidazole residue retains its proton (since it is still 'buried') and is subject to general base-catalysed hydrolysis.

The recent report that the deacylation of *trans*-cinnamoyl-papain is dependent on a basic group with pK 4.69 (Bender & Brubacher, 1964) could be regarded as support for the participation of an imidazole residue in the deacylation process. The relatively non-specific cinnamoyl residue would be expected to lie differently on the enzyme surface and possibly only partially 'bury' the imidazole residue, resulting in its protonation at low pH. That the cinnamoyl-thiol ester bond is poorly orientated with respect to the attacking nucleophile is borne out by the low rate of deacylation (k_{25} , $3.68 \times 10^{-3} \text{sec.}^{-1}$) compared with a specific acyl residue, e.g. hippuryl-papain (k_{35} , 2.7sec.^{-1}) (Lowe & Williams, 1965b).

There is really no evidence that indicates that a carboxylate ion is the nucleophile responsible for the deacylation of acyl-papain, whereas there is now considerable evidence accumulating in favour of an imidazole residue. We tentatively propose that an imidazole residue is the nucleophile involved in this step and are currently seeking more direct evidence to support this view.

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