PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The Structure and Mechanism of Action of Papain

G. Lowe

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The structure and mechanism of action of papain

By G. Lowe
The Dyson Perrins Laboratory, University of Oxford

Introduction

The cysteine proteinases form a group of enzymes which depend for their enzymic activity on the thiol group of a cysteine residue. Several which occur in plants have been investigated extensively and include papain, ficin and stem bromelain (Smith & Kimmel 1960). Although the term papain, introduced last century to describe the proteolytic principle in papaya latex (Wurtz & Bouchut 1879) is still used to describe crude dried latex, the crystalline enzyme is readily obtained (Kimmel & Smith 1954). Ficin is known to consist of several closely related enzymes which have been resolved (Sgarbieri, Gupte, Kramer & Whitaker 1964), but for most structural and mechanistic studies the unresolved mixture of enzymes has been used. Stem bromelain also appears to be a mixture of at least two proteolytic enzymes which have not yet been resolved (Ota, Moore & Stein 1962; Murachi 1964). In spite of the recognized heterogeneity of ficin and stem bromelain, it does seem that both structurally and mechanistically they are similar to papain.

Only one bacterial cysteine proteinase has received a detailed study, namely, streptococcal proteinase, and it appears to have little or no relation in its amino acid sequence with the plant enzymes (Liu, Stein, Moore & Elliott 1965). The functional groups involved in the catalytic mechanism are apparently the same as in the plant proteinases (Gerwin, Stein & Moore 1966; Liu 1967; Husain & Lowe 1968*a*, *c*), but the mechanism of action has not been extensively studied. It may well be however that the plant and bacterial cysteine proteinases have converged onto a similar mechanism of action by two independent evolutionary pathways, as now seems apparent for the animal and bacterial serine proteinases (Alden, Wright & Kraut, this volume, p. 119). Because the tertiary crystal structure of papain (Drenth, Jansonius, Koekoek, Swen & Wolthers 1968; see also the preceding paper, p. 231) is now known, a critical survey of this enzyme is apposite.

Evidence for an essential thiol group in papain

The early realization that papain is a thiol dependent proteinase stemmed from a number of activation and inhibition experiments. Papain is readily inactivated by oxygen, iodine, hydrogen peroxide, and mercuric salts, and reactivated with cyanide ions, hydrogen sulphide and thiols, especially in the presence of EDTA. Papain is irreversibly inhibited however by N-ethylmaleimide and by iodoacetate. The hydrolysate of the enzyme irreversibly inhibited with iodoacetate contained one residue of *S*-carboxymethyl cysteine (Smith & Kimmel 1960; Smith, Light & Kimmel 1962).

Amino acid sequence of papain

Papain is a single chain protein and a tentative amino acid sequence was elucidated by Smith and his collaborators by chemical and enzymic methods (Light, Frater, Kimmel & Smith 1964).

The cysteine residue alkylated by iodoacetate and by the active site directed irreversible inhibitor toluene-*p*-sulphonamidomethyl chloromethyl ketone is Cys-25 (Light *et al.* 1964; Husain & Lowe 1965).

The X-ray crystal structure analysis (Drenth *et al.* 1968) however has revealed that this amino acid sequence requires substantial revision as well as indicating 13 residues from 29 to 41 inclusive (see figure 1 in the preceding paper) which had not been isolated during the degradative studies. A peptide containing the 13 unknown residues has now been obtained consisting of

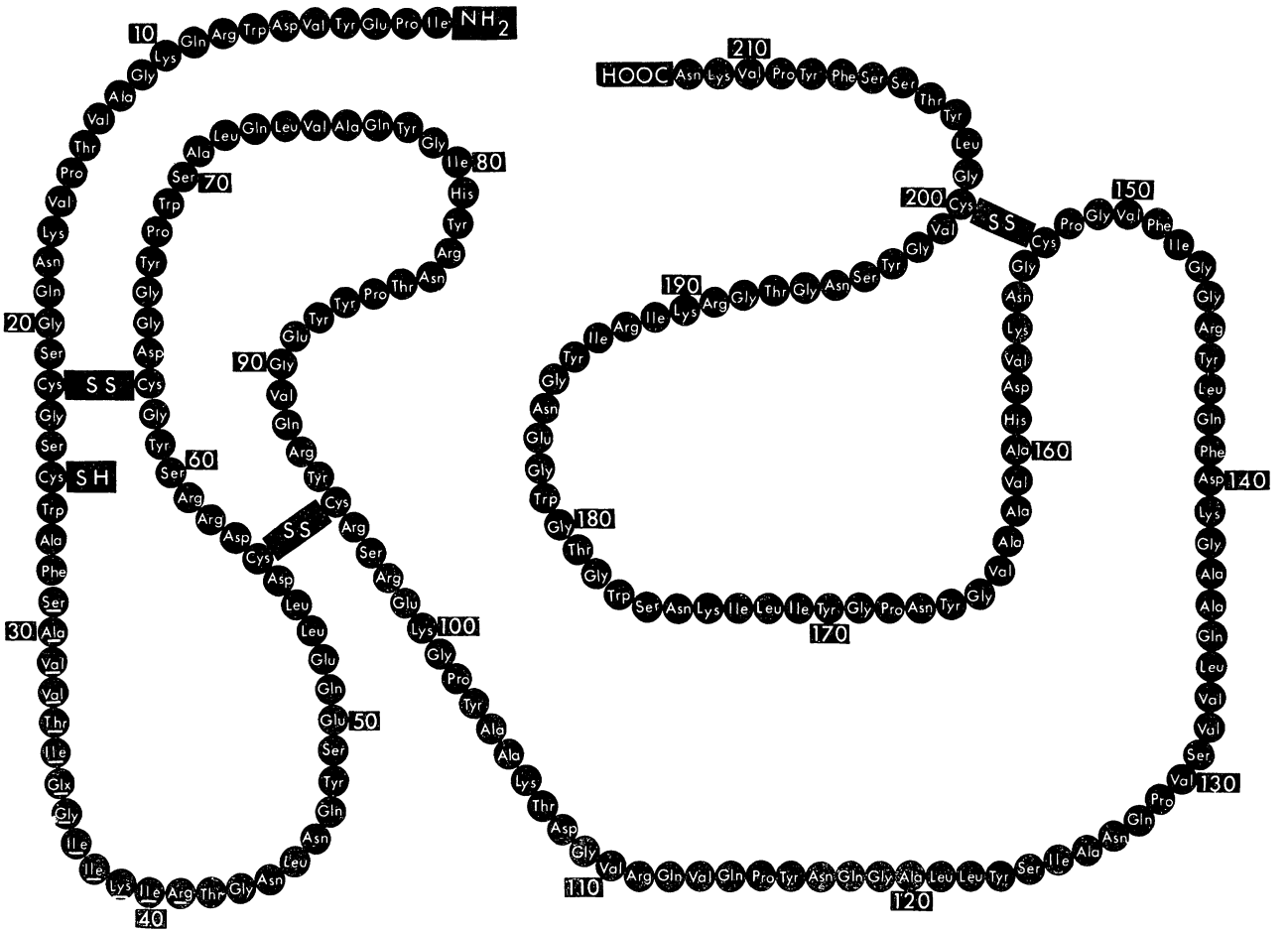

FIGURE 1. The amino acid sequence of papain.

residues 18 to 58 inclusive. The amino acid composition of this peptide, together with the amino acid composition of peptides derived from it by digestion with pepsin and dilute hydrochloric acid, is in agreement with the sequence shown in figure 1 in the preceding paper and establishes the identity of the 13 unknown residues. The amino acid sequence of these residues has been determined (Husain & Lowe 1969) so completing the amino acid sequence of the enzyme (figure 1).†

The active site cysteine remains as residue 25 in the revised sequence (figure 1) so that the claim (Hill & Smith 1955, 1958, 1960) that more than 100 amino acid residues can be removed from the N-terminus of mercuri-papain by leucine aminopeptidase, and that the residual fragment after reactivation retains its full enzymic activity, is untenable.

† Only the amino acid composition of the 13 unknown residues was reported at the meeting.

Mechanism of activation and inactivation of papain

Papain is reversibly inactivated by air in the presence of low concentrations of cysteine and reactivated by high concentrations of cysteine. Using [^{35}S]cysteine it was shown that reversibly inhibited papain is a mixed disulphide of papain and cysteine; the radioactivity is removed on reactivation (Sluyterman 1967). Similar experiments with [^{35}S]cystamine have been reported (Sanner & Pihl 1963). The sedimentation coefficient of reversibly inactivated papain is almost identical with that of activated papain, thus excluding the possibility of an intermolecular disulphide bond between enzyme molecules (Glazer & Smith 1965). This facile activation–inactivation mechanism may provide a simple control mechanism *in vivo*.

Activation of reversibly inactivated papain with radioactive cyanide releases β-thiocyanoalanine, demonstrating a similar mechanism of activation by cyanide ions (Klein & Kirsch 1969). It is surprising therefore that activation of reversibly inactivated papain with [^{35}S]phosphorothioate, gives ^{35}S labelled active enzyme (Neumann, Shinitzky & Smith 1967). It may be that in addition to activating the enzyme, phosphorothioate reacts with an interchain disulphide bond and thereby is incorporated into the protein.

Specificity of papain

Papain exhibits broad specificity for N^{α}-acyl-L-amino acid derivatives. Derivatives of L-arginine and L-lysine are however hydrolysed more rapidly, and those of L-aspartic and L-glutamic acid more slowly than derivatives of uncharged amino acids (Bergmann & Fruton 1941; Smith & Kimmel 1960). It seems probable therefore that the side chain of the substrate is bound to the enzyme close to a carboxylic acid group.

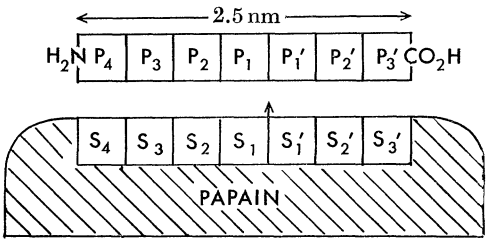

Figure 2. A schematic diagram representing the seven subsites in the active site of papain (Schechter & Berger 1967). Hydrolysis takes place at the peptide bond between amino acid residues bound to subsites S_1 and S_1'.

From an investigation of the rate and pattern of cleavage of 40 diastereoisomeric peptides of alanine, ranging from di- to hexapeptides, it has been suggested that the active site of papain can be regarded as a cleft in the enzyme, made up of seven subsites each capable of accommodating a single amino acid residue of a peptide substrate (Schechter & Berger 1967). The subsites are located on both sides of the catalytic site, four on N-terminal side and three on the C-terminal side of the peptide (see figure 2).

The demonstration of the existence of an active site in papain capable of binding several amino acid residues raises the possibility that single amino acid derivatives which have been most commonly used for kinetic studies with this enzyme may be multiply bound to the active site. This possibility could lead to non-productive binding which would complicate the analysis of kinetic data. Schechter & Berger (1968) have shown that peptides containing L-phenylalanine

are hydrolysed by papain at the peptide bond next-but-one to it towards the C-terminal, that is the phenylalanyl residue is bound specifically to the S_2 subsite (figure 2). This is in accord with the fact that *N*-benzyloxycarbonyl- and *N*-benzoyl-amino-acid derivatives are better substrates for papain than the corresponding *N*-acetyl-amino-acid derivative (table 1). The structural similarity between the phenylalanyl and benzyloxycarbonyl derivatives is particularly clear (figure 3). This probably ensures that substrates with the *N*-benzyloxycarbonyl- and the *N*-benzoyl residues are bound predominantly if not exclusively in the productive mode.

TABLE 1. THE MICHAELIS–MENTEN PARAMETERS FOR THE PAPAIN CATALYSED HYDROLYSIS OF SOME *N*-ACYL-GLYCINE *p*-NITROPHENYL ESTERS AT pH 6.0

substrate	temp/°C	K_m/μmol l^{-1}	k_{cat}/s^{-1}	$\frac{k_{cat}}{K_m}\Big/\frac{s^{-1}}{mol\ l^{-1}}$	reference
Z-Gly	25	8.3 ± 0.6	5.2 ± 0.5	624000	Williams & Whitaker (1967)
Bz-Gly	35	14 ± 3	4.1 ± 0.5	293000	Lowe & Williams (1965*c*)
Ac-Gly	35	1400 ± 200	2.5 ± 0.3	1800	Lowe & Lucas, unpublished results

k_{cat} data are relative to that for N^{α}-benzoyl-L-arginine ethyl ester (Bender *et al.* 1966). Z = $C_6H_5CH_2.O.CO$; Bz = $C_6H_5.CO$; Ac = $CH_3.CO$.

Using poly-glycinamides as nucleophilic acceptors for the deacylation of cinnamoyl-papain, a progressive increase in the rate of deacylation was observed as the number of amino acid residues increased up to the tetrapeptide (Brubacher & Bender 1967). Since the nucleophilic character of the amino group should not be affected this most probably reflects a progressive increase in binding energy as expected from the model of Schechter & Berger (1967) (figure 2).

$$C_6H_5.CH_2.\underset{\underset{NHR}{|}}{\overset{H}{\overset{|}{C}}}.CO.NH.CH_2.COX \quad \text{L-Phe-Gly}$$

$$C_6H_5.CH_2.O.CO.NH.CH_2.COX \quad \text{Z-Gly}$$

$$C_6H_5.CO.NH.CH_2.COX \quad \text{Bz-Gly}$$

$$CH_3.CO.NH.CH_2.COX \quad \text{Ac-Gly}$$

FIGURE 3. Comparison of the structure of L-phenylalanylglycyl derivatives with those of some acylglycyl derivatives.

THE REACTION PATHWAY

The papain catalysed hydrolysis of many derivatives of N^{α}-acyl-L-amino-acids have been shown to obey Michaelis–Menten kinetics. The similar catalytic constants observed for N^{α}-benzoyl-L-argininamide and N^{α}-benzoyl-L-arginine ethyl ester led to the suggestion that an acyl-enzyme intermediate was formed through the essential thiol group and that for these substrates deacylation of this intermediate was rate determining (Stockell & Smith 1957; Smith & Parker 1958). Although it is now known that for these substrates deacylation is not rate determining (Sluyterman 1968) there is much evidence to support the acyl-enzyme hypothesis. The catalytic constants (k_{cat}) for the papain catalysed hydrolysis of aryl and alkyl esters of hippuric acid are virtually identical (Lowe & Williams 1965*c*) as are those of *N*-benzyloxycarbonyl-glycine (Kirsch & Igelström 1966) (table 2), providing evidence for a common step and possibly a common intermediate. Bender *et al.* (1966) have found that papain can be 'titrated' with *N*-benzoyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester at pH 3.2, the initial

TABLE 2. THE MICHAELIS–MENTEN PARAMETERS FOR THE PAPAIN CATALYSED HYDROLYSIS OF SOME *N*-ACYLGLYCINE ESTERS

substrate	temp/°C	pH	K_m/μmol l^{-1}	k_{cat}/s^{-1}	$\frac{k_{cat}}{K_m}\Big/\frac{s^{-1}}{mol\ l^{-1}}$	reference
Bz-Gly-pnp	35	6.0	14 ± 3	2.31 ± .26	171000	Lowe & Williams (1965*c*)
Bz-Gly-Me	35	6.0	20500 ± 2000	2.72 ± .06	133	
Z-Gly-pnp	25	6.8	9.5 ± 1	2.73 ± .08	287000	Kirsch & Igelström (1966)
Z-Gly-Me	25	6.8	5140 ± 740	1.96 ± 14	382	

'burst' of *p*-nitrophenol liberated being proportional to the active enzyme concentration. The most direct evidence, however, came from spectroscopic observation of the acyl-enzyme intermediate from the specific substrate methyl thionohippurate (Lowe & Williams 1965*a*) and the non-specific substrate cinnamoylimidazole (Brubacher & Bender 1966). The wavelength and extinction of the ultraviolet absorption spectrum of the thionohippuryl-papain indicated that it was a dithioester presumably formed through the active site thiol group. The change in extinction of the absorption band with time was as expected from the Michaelis–Menten parameters for methyl thionohippurate determined independently, assuming deacylation to be the rate determining step. With cinnamoylimidazole it was possible to isolate cinnamoyl-papain and study its deacylation independently. The wavelength and extinction of the ultraviolet absorption spectrum indicated that a thioester was formed presumably through the active site thiol group. The reaction pathway can therefore be written as:

$$E + S \overset{K_s}{\rightleftarrows} ES \overset{k_2}{\rightarrow} ES' \overset{k_3}{\rightarrow} E + P_2 + P_1$$

where E is the enzyme, S the substrate, ES the enzyme–substrate complex, ES′ the acyl-thiolenzyme intermediate, P_1 the amine, ammonia or alcohol and P_2 the carboxylic acid derived from the substrate. By studying the pre-steady state kinetics of some esters of *N*-benzyloxycarbonyl-glycine, Hubbard & Kirsch (1968) confirmed that the ratio of the observed Michaelis–Menten parameters, $k_{cat}/K_m = k_2/K_s$ at pH 6.8.

pH DEPENDENCE OF THE MICHAELIS–MENTEN PARAMETERS

The stability of papain over a wide range of pH has allowed the pH dependence of the Michaelis–Menten parameters for several substrates to be determined. It is necessary, however, for a correct interpretation of the pH dependence of the individual steps, to consider substrates for which deacylation is rate determining ($k_2 \gg k_3$), separately from those in which acylation is rate determining ($k_2 \ll k_3$). Furthermore, it is desirable to separate charged from uncharged substrates since charge on the substrate has been shown to influence the pH dependence, for example with *N*-benzyloxycarbonyl-L-histidinamide and *N*-benzyloxycarbonyl-glycyl-glycine (Smith, Chavré & Parker 1958). Finally a distinction between specific and non-specific substrates may also be desirable. Data are presented in this way in table 3.

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TABLE 3. THE pH DEPENDENCE OF THE MICHAELIS–MENTEN PARAMETERS AT 25 °C FOR THE PAPAIN CATALYSED HYDROLYSIS OF SUBSTRATES FOR WHICH $k_2 \gg k_3$.

	k_{cat}/K_m		k_{cat}	
	pK_1	pK_2	pK_1	references
(*a*) uncharged				
(i) specific				
Z-Gly-pnp	4.23	8.50	3.85	Williams & Whitaker (1967)
N^{α}-Z-N^{ϵ}-formyl-L-Lys-pnp	—	—	3.96	Bender & Brubacher (1966)
Ac-DL-Trp-pnp	4.40	—	4.70	
(ii) non-specific				
cinnamoyl	—	—	4.69	Brubacher & Bender (1966)
(*b*) charged and specific				
Z-L-Lys-pnp	4.30	8.0	3.52	Bender & Brubacher (1966)
Z-L-Lys-$CH_2C_6H_5$	4.35	8.35	3.50	

The deacylation step (k_3)

The Michaelis–Menten parameters were determined for *p*-nitrophenyl *N*-benzyloxycarbonyl-glycinate from pH 3.0 to 9.5. For this substrate k_{cat} ($= k_3$) shows pH dependence with an apparent pK of 3.85. For *p*-nitrophenyl N^{α}-benzyloxycarbonyl N^{ϵ}-formyl-L-lysinate k_{cat} is dependent on a group with a similar apparent pK 3.96. Interestingly, however, the pH dependence of k_{cat} for *p*-nitrophenyl N^{α}-acetyl-DL-tryptophanate which almost certainly represents k_3 for this substrate and the deacylation cinnamoyl-papain show dependence on a group with an apparent pK 4.7, some 0.8 pH units higher than the former two substrates. Although the cinnamoyl residue is non-specific, the tryptophan derivative is a specific substrate and it seems unlikely that the presence of the D-enantiomer would cause this pK shift. For the charged substrates *p*-nitrophenyl and benzyl N^{α}-benzyloxycarbonyl-L-lysinate k_{cat} again can be assigned reasonably to k_3, and shows dependence on a group with an apparent pK 3.5. Thus the nature of the substrate influences the pH dependence of deacylation.

The binding and acylation steps

In order to study the pH dependence of these steps, substrates are required in which acylation is rate limiting. The substrates N^{α}-benzoyl-L-arginine ethyl ester and N^{α}-benzoyl-L-arginin-amide are among the most thoroughly studied with this enzyme but have given rise to a very confused literature. Because of the similarity of k_{cat} for the two substrates Smith & Parker (1958) concluded that deacylation was rate determining. Whitaker & Bender (1965), however, suggested that for the ethyl ester, deacylation is essentially rate determining, whereas for the amide, acylation is essentially rate determining. Two experiments, the first involving a study of the rate of activation of papain by cysteine in the presence of the substrate (Sluyterman 1966), and

TABLE 4. THE pH DEPENDENCE OF THE MICHAELIS–MENTEN PARAMETERS FOR THE PAPAIN CATALYSED HYDROLYSIS OF SUBSTRATES FOR WHICH $k_2 \ll k_3$.

	k_{cat}/K_m		k_{cat}			
	pK_1	pK_2	pK_1	pK_2	temp/°C	references
Bz-L-citrulline-Me	4.23	8.50	4	8.5	25	Williams & Whitaker (1967); Cohen & Petra (1967)
Bz-L-Arg-NH_2	4.24	8.35	3.65	8.31	25	Whitaker & Bender (1965)
Bz-L-Arg-Et	4.29	8.49	4.04	9.10	25	

the second involving the determination of the rate of inhibition of papain by chloroacetic acid in the presence of substrate (Sluyterman 1968) have demonstrated that for N^{α}-benzoyl-L-arginine ethyl ester, acylation is the rate determining step. For both these substrates k_{cat} and k_{cat}/K_m show bell shaped pH dependence (Whitaker & Bender 1965) (see table 4).

The uncharged but isosteric substrate N^{α}-benzoyl-L-citrulline methyl ester also shows bell shaped pH dependence for k_{cat} and k_{cat}/K_m (Williams & Whitaker 1967). Although it has not been established that for this substrate acylation (k_2) is the rate determining step, the pH dependence together with the close structural analogy with N^{α}-benzoyl-L-arginine ethyl ester suggests that it probably is. The variation in the apparent pK for k_{cat} for these substrates indicates that the apparent pK of the catalytic groups are perturbed differently by different substrates in the enzyme–substrate complex.

As a result of pH dependence studies it has generally been concluded that papain depends on a carboxylic acid group in addition to the essential thiol group for its enzymic activity. The insensitivity of the apparent pK to temperature of the acid limb for k_{cat}/K_m against pH for the papain catalysed hydrolysis of N^{α}-benzoyl-L-arginine ethyl ester (Smith & Parker 1958) has been regarded by many as confirmatory evidence for this conclusion since carboxylic acid groups generally have small or zero heats of ionization. This criterion, however, must be regarded as an unreliable guide to the identity of the functional group, since the apparent pK determined from the pH dependence of k_{cat}/K_m for benzoylglycinamide rises with temperature leading to an apparent negative heat of ionization to which no group can be assigned (Smith *et al.* 1958). A similar apparent increase in pK with temperature is observed for the pH dependence of k_{cat} for N^{α}-benzoyl-L-arginine ethyl ester (Smith & Parker 1958).

pH *dependence of irreversible inhibition*

The pH dependence of irreversible inhibition of papain by alkylating reagents is particularly instructive since this involves a different type of reaction namely nucleophilic substitution at the catalytic site. The inhibition of papain by the chloromethylketone derived from *N*-toluene-*p*-sulphonyl-L-phenylalanine (TPCK) shows sigmoid pH dependence with apparent pK 8.28, the rate of inhibition rising with pH (Bender & Brubacher 1966). The alkylation of papain by D(+)- and L(−)-α-iodopropionamide also shows sigmoid pH dependence with apparent pK 8.0, whereas inhibition of papain by L(−)-α-iodopropionic acid shows bell shaped pH dependence with apparent pK 4.0 and 7.8 (Wallenfels & Eisele 1968). All three reagents react with the active site cysteine residue. The pH dependence for the inhibition of papain by L(−)-α-iodopropionic acid is very similar to the pH dependence of k_{cat}/K_m for substrate hydrolysis, but that of uncharged alkylating agents depends on the deprotonated form of the active enzyme. This is entirely reasonable since nucleophilic substitution of iodide ions depends only on the nucleophile and is unaffected by general acids, whereas ester and amide hydrolysis are susceptible to both nucleophilic (or general base) and general acid catalysis. The bell shaped pH dependence for L(−)-α-iodopropionic acid presumably arises because of the charge on this inhibitor (in the pH range investigated) is attracted by a positive charge on the enzyme. This is not consistent with the presence of a carboxylic acid group in the catalytic site.

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Evidence for an imidazole group in the active site of papain

A study of the intramolecular carboxylate ion catalysed hydrolysis of *S*-hippuryl-thioglycollic acid and ethyl thiolsuccinate indicated that the carboxylic acid group was an insufficiently effective catalyst to account for the deacylation of acyl-papains. The imidazole group, however, was a much more effective catalyst and capable of accounting for the observed rates of deacylation (Lowe & Williams 1965*b*).

Direct evidence for the propinquity of an imidazole group to the active site thiol group was provided using the bifunctional irreversible inhibitor 1,3-dibromoacetone (Husain & Lowe 1968*a*). Since the active site cysteine residue is alkylated by iodoacetate, 1,3-dibromoacetone was expected first to alkylate Cys-25 and subsequently to alkylate a second nucleophile within a radius of 0.5 nm. The hydrolysate of performic acid oxidized papain which had been inhibited with [2–^{14}C]dibromoacetone contained *S*-carboxymethyl cysteine sulphone and 1-carboxylmethyl histidine as the only radioactive residues. Enzymic degradation of the inhibited enzyme and isolation of the radioactive peptide showed that Cys-25 and His-159 (residues 106 in the tentative sequence of Light *et al.* 1964) had been crosslinked (Husain & Lowe 1968*b*). The nearest nitrogen of the imidazole group of His-159 must be therefore within a range of 0.5 nm of the active site thiol group and may even be hydrogen bonded to it. This conclusion is confirmed by the crystal structure, which also shows that the nearest carboxylic acid residues are those of Asp-64 (residue 163 in the tentative sequence of Light *et al.* 1964) and Asp-158 (residue 105 in the tentative sequence of Light *et al.* 1964) which are both 1.0 nm from the active site thiol group (Drenth *et al.* 1968).

The mechanism of action of papain

A knowledge of some if not all of the intermediates along the reaction pathway and the nature of the functional groups involved in catalysis, provides a foundation on which to discuss the mechanism of action of papain. The specificity of the enzyme delineates the essential requirements for binding at the active site and at present there is no evidence for distortion of bond angle or bond lengths of the enzyme bound substrate, although conformational constraint may be expected.

Acylation

From the bell shaped pH dependence of acylation and the functional groups involved in catalysis a thiol-imidazole system or its kinetic equivalent appears to represent the active enzyme, which on protonation or deprotonation becomes inactive. Hydrogen bonding of the thiol-imidazole system would of course lead to a high degree of polarization. In the enzyme substrate complex further polarization may be induced by the ester or amide bond of the substrate.

From a study of the papain catalysed hydrolysis of hippuric anilides for which the acylation step (k_2) is rate determining, the binding (K_s) and acylation (k_2) constants can be determined. The Hammett plot for K_s against σ was somewhat scattered. The Hammett plot for acylation gave $\rho = -1.0$ (figure 4) clearly indicating the importance of electrophilic, presumably general acid, catalysis in the acylation step.

The Hammett plot for k_{cat}/K_m for papain catalysed hydrolysis of aryl hippurates gives $\rho = +1.2$ (figure 5) (Lowe & Williams 1965*c*), whereas for the thiolate ion catalysed hydrolysis of aryl *N*-benzyloxycarbonyl-glycinates $\rho = +1.9$ (calculated from data of Kirsch &

Igelström 1966). However for these substrates k_{cat}/K_m is a composite term involving binding and acylation making interpretation more difficult. The less positive ρ value for the enzyme reaction compared with the thiolate ion catalysis however could be due to general base rather than specific base catalysis.

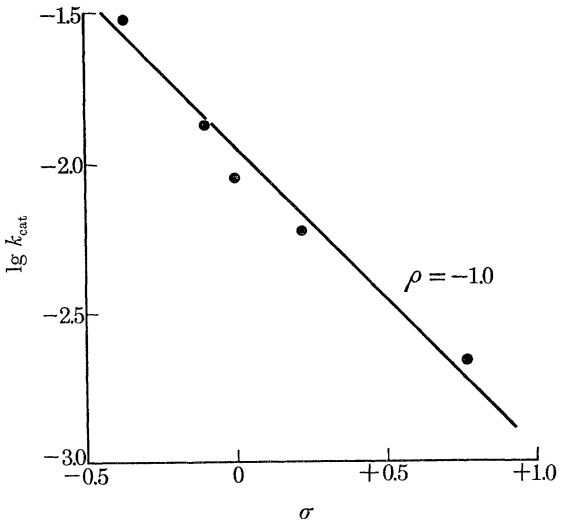

FIGURE 4. A Hammett plot of the catalytic constant (representing acylation) for papain catalysed hydrolysis of some *p*-substituted hippuryl anilides (G. Lowe & Y. Yuthavong unpublished results).

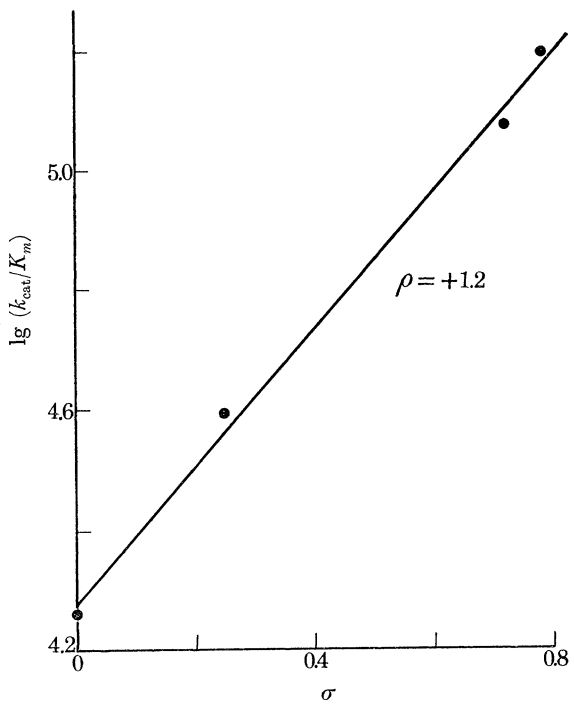

FIGURE 5. A Hammett plot for the papain-catalysed hydrolysis of some *p*-substituted aryl hippurates (Lowe & Williams 1965*c*).

Deacylation

Papain catalysed ^{18}O exchange from $H_2{}^{18}O$ into several virtual substrates (e.g. N^{α}-benzyloxycarbonyl-L-arginine, -L-tyrosine, -L-tryptophan and -glycine) suggests that the principle of microscopic reversibility is applicable to papain catalysed hydrolyses (Grisaro & Sharon 1964). Therefore deacylation should be general base catalysed by an imidazole group. Evidence that deacylation of benzyloxycarbonyl-glycyl-papain (Henry & Kirsch 1967) and cinnamoyl-papain (Brubacher & Bender 1966) are general base catalysed has been presented. That the catalytic group is indeed an imidazole group is suggested by the solvent dependence of the apparent pK

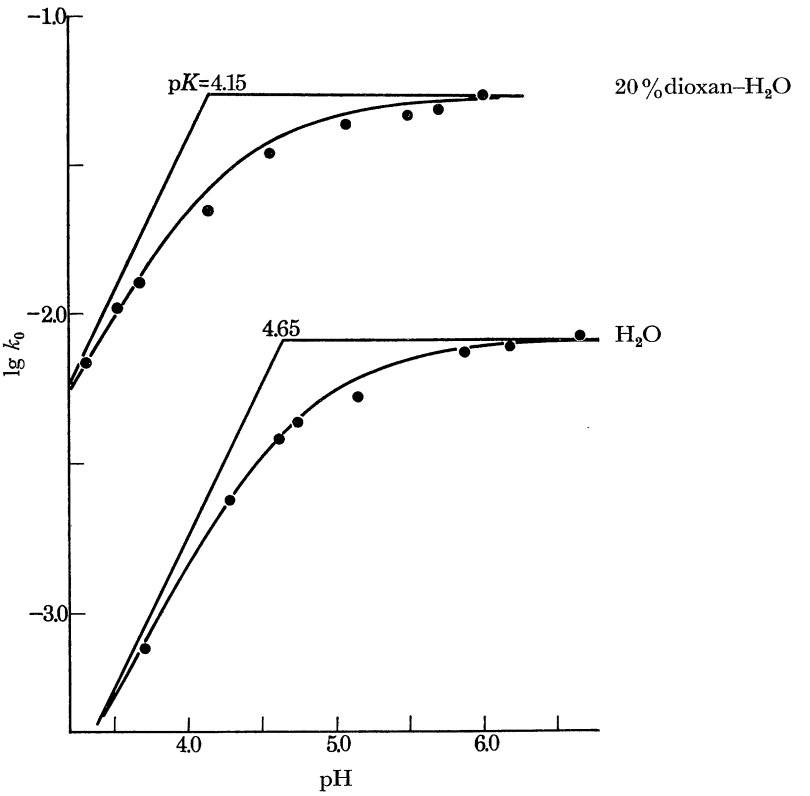

FIGURE 6. The dielectric effect of the solvent on the apparent pK for the deacylation of cinnamoyl-papain (G. Allen & G. Lowe unpublished results).

of the group involved in deacylation. The pK of an uncharged acid such as a carboxylic acid is strongly dependent on the dielectric constant of the solvent since two charges are generated from an uncharged species on ionization. The pK increases with a lowering of the dielectric constant of the medium. The pK of positively charged acids such as the imidazolium ion are rather less sensitive to the solvent, the pK decreasing in a solvent of lower dielectric constant (Mandel 1958; Findlay, Mathias & Rabin 1962). The solvent dependence of the apparent pK for the deacylation of cinnamoyl-papain is shown in figure 6 (G. Allen & G. Lowe, unpublished results). The pK of 4.65 in water moves to 4.15 in 20 % dioxan–water which must be assigned to a positively charged acid for which imidazole is the only possibility. In table 5 these data are compared with the pK of hippuric acid and 4(5)-bromoimidazole in water and 20 % dioxan–water.

TABLE 5. THE DIELECTRIC EFFECT OF SOLVENT ON SOME pK AT 35 °C

	pK(H_2O)	pK(20% dioxan–H_2O)	ΔpK
hippuric acid	3.65	4.10	+0.45
4-bromoimidazole	3.65	3.32	−0.33
cinnamoyl-papain	4.65	4.15	−0.50

CONCLUDING REMARKS

Although it does appear that catalysis by papain is at any rate in part promoted by the interplay of the active site thiol and imidazole groups with the substrate, in both the acylation and deacylation steps it would be reasonable to assume, by analogy with simple ester and amide hydrolysis, that tetrahedral intermediates are involved, although no direct evidence for their intervention is available. Since the hydrolytic step in deacylation (now assumed to be the formation of a tetrahedral intermediate) appears to be general base catalysed, its breakdown

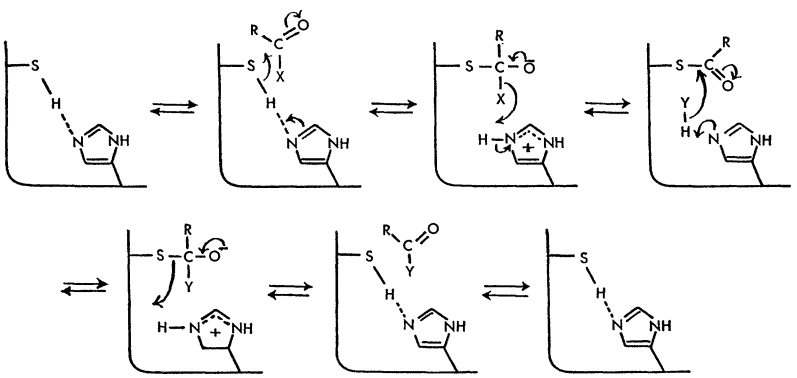

FIGURE 7. A possible mechanism of action for papain catalysed hydrolyses.

must be general acid catalysed. Applying the same argument to acylation (which is valid if the principle of microscopic reversibility is applicable) the formation of the tetrahedral intermediate will be general base catalysed and its breakdown general acid catalysed. This mechanism accounts for the widely differing ρ values for hippuryl anilides (figure 4) and aryl hippurates (figure 5). If with hippuryl anilides the breakdown of the tetrahedral intermediate to the acyl-enzyme (ES′) is slow compared with the breakdown to enzyme–substrate complex (ES) the observed acylation constant will be a composite constant involving the rate constants for the formation and breakdown of the tetrahedral intermediate. This is of course very probable with anilides since the anilines are relatively poor leaving groups. With aryl hippurates however since phenols are good leaving groups it is probable that the breakdown of the tetrahedral intermediate to the acyl-enzyme (ES′) is fast compared with the breakdown to the enzyme–substrate complex (ES) so that the acylation constant represents the formation of the tetrahedral intermediate. Since general base catalysis would give rise to a positive ρ value and general acid catalysis to a negative ρ value, it is possible to explain the observed ρ values for hippuryl anilides and aryl hippurates by incorporating a tetrahedral intermediate into the reaction scheme.

Recognizing that kinetically equivalent mechanisms cannot be excluded, that a reaction mechanism is subject to further refinement and that conformational changes may take place in the enzyme and substrate along the reaction pathway, a possible mechanism of action of papain is proposed (figure 7) which accounts for the available experimental evidence.

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