Kinetic Specificity in Papain-Catalysed Hydrolyses

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The specificity of the proteolytic enzyme, papain, for the peptide bond of the substrate adjacent to that about to be cleaved and for the acyl residue of some *N*-acylglycine derivatives is manifest almost exclusively in the formation of the acyl-enzyme from the enzyme–substrate complex. Models for the enzyme–substrate complex and acyl-enzyme intermediate are suggested that account for these observations. In particular it is suggested that the peptide bond of the substrate adjacent to that about to be cleaved, is bound in the cleft of the enzyme between the NH group of glycine-66 and the backbone C=O group of aspartic acid-158, and provides a sensitive amplification mechanism through which the specificity of the enzyme for hydrophobic amino acids such as L-phenylalanine is relayed. It is also suggested that the distortion in the enzyme–substrate complex and the binding of the peptide bond adjacent to that about to be cleaved are also linked and behave co-operatively, the distortion of the protein facilitating binding and the stronger binding facilitating distortion. The results imply that between the enzyme–substrate complex and the acyl-enzyme a relaxation of the protein conformation must occur.

There is now available considerable evidence that papain-catalysed hydrolyses proceed by a reaction pathway involving at least three steps (Lowe, 1970, for a recent summary of the evidence),

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \overset{k_{+2}}{\longrightarrow} ES' \overset{k_{+3}}{\longrightarrow} E + P_2$$
$$+ P_1$$
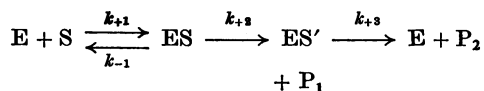
where ES is the Michaelis complex, ES' is the acyl-enzyme formed through the thiol group of cysteine-25, P_2 is the acid and P_1 the alcohol or amine moiety of the hydrolysed substrate. The Michaelis–Menten parameters, $k_{cat.}$ and K_m obtained from the steady-state reaction rates according to the equation

$$v_0 = k_{cat.}[E][S_0]/(K_m + [S_0])$$

are related to the rate constants in the three-step reaction pathway as follows:

$$k_{cat.} = k_{+2}k_{+3}/(k_{+2} + k_{+3})$$

and

$$K_m = k_{+3}(k_{-1} + k_{+2})/k_{+1}(k_{+2} + k_{+3})$$

If deacylation (k_{+3}) is the rate-determining step then $k_{cat.} = k_{+3}$ and if $k_{-1} \gg k_{+2}$, $K_m = k_{-1}k_{+3}/k_{+1}k_{+2} = K_s k_{+3}/k_{+2}$. If, however, acylation (k_{+2}) is the rate-determining step then $k_{cat.} = k_{+2}$ and, if $k_{-1} \gg k_{+2}$, $K_m = k_{+1}/k_{+1} = K_s$.

The specificity of an enzyme stems from its ability to bind some substances more effectively than others, but it is nevertheless frequently observed that the catalytic rate constants reflect the specificity of an enzyme, that is kinetic specificity is apparent. In the simplest case, however, specificity is manifest in the binding equilibrium constant, K_s, and then specificity and catalysis are clearly separated. Kinetic specificity arises when the binding equilibrium constant, K_s, and the catalytic constant(s) are mutually linked so that free energy lost to binding is gained by catalysis (see Jencks 1969, for discussion). In a multi-step reaction pathway, the specificity of the enzyme may be manifest in any step or combination of steps.

Until the work of Berger & Schechter (1970), papain was regarded as having rather broad specificity for L-amino acid derivatives, showing a slight preference for derivatives of basic amino acids. Berger & Schechter (1970), however, showed that the active site of papain could accommodate a peptide sequence of up to seven amino acid residues, and the presence of a hydrophobic residue such as L-phenylalanine or L-tyrosine or to a less extent L-leucine, directed the enzymic cleavage not to its own peptide bond (as in α-chymotrypsin) but to the bond of the adjacent *C*-terminal residue, except when this was L-valine. This conclusion

which was based on the specificity of papain for some synthetic peptides, was supported by an investigation of the sites of cleavage of the oxidized B chain of insulin (Johansen & Ottesen, 1968).

As a result of their observations Berger & Schechter (1970) proposed a model for the binding of a peptide to papain based on the tertiary structure of the enzyme. Their model, however, failed to account for several important observations reported in the literature, and they themselves expressed doubts as to its validity.

By the use of steady-state kinetic methods and a combination of substrates, the specificity of papain for the acyl residue in some N-acylglycine derivatives and the peptide bond adjacent to that which is cleaved, has been explored. These observations combined with a knowledge of the tertiary structure of papain (Drenth, Jansonius, Koekoek, Swen & Wolthers, 1968) have led to a plausible model for the acyl-enzyme and the enzyme–substrate complex that is in substantial agreement with that independently proposed by Wolthers, Drenth, Jansonius, Koekoek & Swen (1970), on the basis of preliminary crystallographic evidence.

MATERIALS AND METHODS

Papain. Twice-crystallized papain was obtained from dried papaya latex (we acknowledge a gift of this material from the Wallerstein Co., New York, N.Y., U.S.A.), by the method of Kimmel & Smith (1954) and was activated with p-thiocresol (Soejima & Shimura, 1961). The enzyme concentration was determined by using methyl hippurate as substrate and the data of Lowe & Williams (1965).

p-*Nitrophenyl hippurate.* This was prepared as described by Lowe & Williams (1965) and had m.p. 171–172.5°C (lit. m.p. 170–171°C).

p-*Nitrophenyl 3-benzoylpropionate.* 3-Benzoylpropionic acid (3.56g) prepared by the method of Somerville & Allen (1933), and triethylamine (2.0g) were dissolved in chloroform (20ml) and cooled to −5°C. Ethyl chloroformate (2.2g) was added dropwise over 10min to the stirred solution. A solution of p-nitrophenol (2.8g) and triethylamine (2.0g) in chloroform (10ml) was added dropwise over 10min and the mixture was stirred in the cold for a further 30min, at room temperature for 2h, and at 40°C for 15min. The solution was washed with 2M-HCl (50ml), saturated Na_2CO_3 solution (50ml) and water, and dried over anhydrous Na_2SO_4. Platelets of p-*nitrophenyl 3-benzoylpropionate* were obtained on addition of light petroleum (b.p. 60–80°C); yield, 2.4g (40%). The product was recrystallized from acetone–water, and from benzene; m.p. 124–126°C (decomp.); $\lambda_{max.}$ (in ethanol), 244nm (ϵ 15800), 270nm (ϵ 10400) (Found: C, 64.2; H, 4.3; N, 4.7; $C_{16}H_{13}NO_5$ requires C, 64.25; H, 4.35; N, 4.7%).

p-*Nitrophenyl 4-phenylbutyrate.* 4-Phenylbutyric acid (1.64g) (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) and p-nitrophenol (1.4g) were dissolved in ethyl acetate (30ml) at 0°C. NN'-Dicyclohexylcarbodi-imide

(2.1g) was added and the mixture was stirred overnight, the solution being allowed to warm up gradually to room temperature. The precipitate was filtered off and the filtrate was concentrated *in vacuo*. The residue, after recrystallizations from ether–light petroleum (b.p. 60–80°C) gave platelets of p-*nitrophenyl 4-phenylbutyrate*; yield, 2.1g (73%); m.p. 43–44°C; $\lambda_{max.}$ (in ethanol) 269nm (ϵ 7200) (Found: C, 67.35; H, 5.5; N, 4.6; $C_{16}H_{15}NO_4$ requires C, 67.4; H, 5.3; N, 4.9%).

Hippuryl p-*anisidide.* To a suspension of powdered hippuric acid (3.6g) in methylene chloride (10ml) was added triethylamine (2g) and the clear solution formed was stirred and cooled to about −10°C, the mixture being protected from atmospheric moisture. A solution of ethyl chloroformate (2.2g) in methylene chloride (10ml) was added dropwise over 15min. After a further 15min a solution of p-anisidine (2.5g) in methylene chloride (15ml) was added dropwise over 30min to the stirred solution maintained at −5°C. Stirring was continued for 1h, after which the white precipitate of *hippuryl* p-*anisidide* was filtered off, washed with saturated $NaHCO_3$ solution and water, and recrystallized from acetone–water; yield, 3g (50%); m.p. 210–212°C; $\lambda_{max.}$ (in ethanol) 249nm (ϵ 20000) (Found: C, 68.4; H, 5.7; N, 9.6; $C_{16}H_{16}N_2O_3$ requires C, 68.7; H, 5.6; N, 9.8%).

3-*Benzoylpropionyl* p-*anisidide.* This was prepared by the same method as that described for hippuryl p-anisidide. 3-Benzoylpropionic acid (0.88g), prepared by the method of Somerville & Allen (1933), and equivalent amounts of p-anisidine (0.61g) and other reagents were used; crude yield of 3-*benzoylpropionyl* p-*anisidide*, 1.39g (98%). The product was recrystallized from acetone–light petroleum (b.p. 60–80°C); m.p. 125–126.5°C; $\lambda_{max.}$ (in ethanol) 248nm (ϵ 22000) (Found: C, 72.0; H, 6.1; N, 5.0; $C_{17}H_{17}NO_3$ requires C, 72.0; H, 6.0; N, 4.9%).

4-*Phenylbutyryl* p-*anisidide.* This was prepared by the same method as that described for hippuryl p-anisidide. 4-Phenylbutyric acid (1.64g) (Koch–Light Laboratories Ltd.) and equivalent amounts of p-anisidine (1.2g) and other reagents were used; crude yield of 4-*phenylbutyryl* p-*anisidide*, 2.45g (92%). The compound was recrystallized from methanol–water; m.p. 97–98°C; $\lambda_{max.}$ (in ethanol) 251nm (ϵ 13400) (Found: C, 75.6; H, 7.3; N, 5.25; $C_{17}H_{19}NO_2$ requires C, 75.8; H, 7.1; N, 5.2%).

N-*Acetylglycine* p-*nitroanilide.* Powdered N-acetylglycine (1.3g) and PCl_5 (2.3g) in anhydrous ether (40ml) were stirred at 0°C for 1h. p-Nitroaniline (1.5g) in anhydrous ether (50ml) was added and the mixture stirred at 0°C for 3h. The yellow precipitate was filtered off, washed with light petroleum (b.p. 60–80°C), ethanol, saturated $NaHCO_3$ solution and water. The crude N-*acetylglycine* p-*nitroanilide* (0.8g) was recrystallized three times from ethanol, m.p. 262–264°C; $\lambda_{max.}$ (in ethanol) 222nm (ϵ 5100) and 313nm (ϵ 7200) (Found: C, 50.8; H, 4.8; N, 17.9; $C_{10}H_{11}N_3O_4$ requires C, 50.7; H, 4.7; N, 17.7%).

Hippuryl p-*nitroanilide.* Powdered hippuric acid (2g) and PCl_5 (2.4g) in anhydrous ether (40ml) were stirred at 0°C for 1h. A solution of p-nitroaniline (1.5g) in anhydrous ether (75ml) was added dropwise over 10min. The mixture was stirred for 4h at 0°C and then for 16h at 18°C. The precipitate was filtered off and washed with ether, saturated $NaHCO_3$ solution and water. The crude *hippuryl* p-*nitroanilide* (1.7g) was recrystallized from

ethanol as pale-yellow needles, m.p. 248–250°C; $\lambda_{max.}$ (in water containing 8% CH_3CN) 231 nm (ϵ 13300), 317 nm (ϵ 10000) (Found: C, 60.4; H, 4.3; N, 1.40; $C_{15}H_{13}N_3O_4$ requires C, 60.2; H, 4.4; N, 14.0%).

N-*Acetyl*-L-*phenylalanylglycine* p-*nitroanilide.* This was prepared by the same method as that described for hippuryl p-anisidide. Acetyl-L-phenylalanylglycine (0.66 g) and equivalent amounts of p-nitroaniline (0.35 g) and other reagents were used; crude yield of *acetyl*-L-*phenyl-alanylglycine* p-*nitroanilide,* 0.52 g (54%). The product was recrystallized several times from acetone–ether–light petroleum (b.p. 60–80°C); m.p. 221–223°C; $[\alpha]_D^{20}$ +117° (c 0.3 in dimethylformamide); $\lambda_{max.}$ (in ethanol) 313 nm (ϵ 13300) (Found: C, 59.4; H, 5.4; N, 14.4; $C_{19}H_{20}N_4O_5$ requires C, 59.3; H, 5.25; N, 14.6%).

N-*Acetylglycine methyl ester.* This was prepared by the method of Curtius & Goebel (1888) and had m.p. 55–56°C (lit. m.p. 57–58.7°C).

Methyl hippurate. This was prepared as described by Lowe & Williams (1965) and had m.p. 81–83°C (lit. m.p. 81°C).

N-*Acetyl*-L-*phenylalanylglycine methyl ester.* This was prepared by the method of Knowles, Sharp & Greenwell (1969) and had m.p. 141–142°C, $[\alpha]_D^{20}$ −12.6° (c 1.0 in dimethylformamide) (lit. m.p. 144.5–146°C).

N-*Acetylglycine* p-*nitrophenyl ester.* This was prepared by the method of Bryce, Roeske & Gurd (1965) and had m.p. 123–125°C (lit. m.p. 123–125°C).

Acetyl-L-*phenylalanylglycine* p-*nitrophenyl ester.* Acetyl-L-phenylalanylglycine (0.2 g) prepared by the method of Knowles *et al.* (1969) was dissolved in warm ethyl acetate (30 ml). The solution was cooled to 0°C and p-nitrophenol (0.105 g) and NN'-dicyclohexylcarbodi-imide (0.15 g) were added successively. The mixture was stirred at 0°C for 30 min and at 18°C for 5 h. The precipitate was filtered off and the filtrate evaporated *in vacuo.* From the residual syrup, *acetyl*-L-*phenylalanylglycine* p-*nitrophenyl ester* was obtained as colourless crystals (0.11 g) from acetone–ether, m.p. 192–193°C; $[\alpha]_D^{20}$ −9.1° (c 0.3 in dimethylformamide); $\lambda_{max.}$ (in ethanol) 269 nm (ϵ 8700) (Found: C, 59.1; H, 5.0; N, 10.8; $C_{19}H_{19}N_3O_6$ requires C, 59.2; H, 5.0; N, 10.9%).

N-*Acetylaminoacetonitrile* (with G. Allen). This was prepared by the method of Johnson & Gatewood (1929) and had m.p. 75–78°C (lit. m.p. 77°C).

N-*Acetylthioglycine amide* (with G. Allen). This was prepared by the method of Johnson & Gatewood (1929) and after elution with ethanol on silica gel (M60; Harrington Bros. Ltd., London E.C.1, U.K.) and re-crystallization from ethanol gave colourless plates, m.p. 124–126°C (lit. m.p. 123–124°C).

Hippuronitrile. This was prepared by the method of Klages & Haack (1903). It was crystallized from ethanol as white plates, m.p. 143–144°C (lit. m.p. 144°C).

Thiohippurylamide. (with E. D. Sinclair). This was prepared by the method of Johnson & Burnham (1912) and crystallized from aqueous ethanol as colourless needles m.p. about 150°C (decomp.) (lit. m.p. 150°C decomp.) (Found: C, 55.3; H, 5.0; N, 14.3; S, 16.5; Calc. for $C_9H_{10}N_2OS$: C, 55.6; H, 5.2; N, 14.4; S, 16.5%).

N-*Methoxycarbonyl*-L-*phenylalanylaminoacetonitrile.* N-Methoxycarbonyl-L-phenylalanine (10.6 g) and triethylamine (5.0 g) were dissolved in methylene chloride (100 ml). The solution was stirred at −5°C while a solution of ethyl

chloroformate (5.4 g) in methylene chloride (20 ml) was added over 15 min. After a further 15 min a solution of aminoacetonitrile bisulphate (7.7 g, Koch–Light Laboratories Ltd.) adjusted to pH 7 with 2M-NaOH, was added over 30 min, the temperature being maintained at −5°C. The mixture was stirred for 18 h and allowed to warm up gradually to room temperature. The *nitrile* (7.5 g) was filtered off and recrystallized from methylene chloride; m.p. 174–175°C; $[\alpha]_{365}^{20}$ +19.3° (c 0.5 in ethanol) (Found: C, 59.5; H, 5.8; N, 15.7; $C_{13}H_{15}N_3O_3$ requires C, 59.8; H, 5.8; N, 16.1%).

N-*Methoxycarbonyl*-L-*phenylalanylglycine thioamide.* The above nitrile (2.0 g) was dissolved in a mixture of dry pyridine (5 ml) and triethylamine (3 ml). Dry H_2S was passed through the solution at 18°C for 90 min, and the solution was kept in a closed vessel for a further 60 min. The solution was poured into water (100 ml), and the product extracted with chloroform (3×100 ml). The *thioamide* (0.9 g) was crystallized from chloroform–light petroleum (b.p. 60–80°C). It was purified by column chromatography on silica gel with ether as eluent and recrystallized from chloroform–light petroleum (b.p. 60–80°C); m.p. 139–141°C; $[\alpha]_D^{20}$ +30° (c 1 in ethanol); $\lambda_{max.}$ (in ethanol) 267 nm (ϵ 14000) (Found: C, 52.6; H, 5.8; N, 14.1; S, 11.0; $C_{13}H_{17}N_3O_3S$ requires C, 52.8; H, 5.8; N, 14.3; S, 10.9%).

Kinetics. All solutions contained 1 mM-EDTA and were 0.05–0.3M with respect to NaCl and/or buffer ions as indicated. All hydrolyses were performed at pH 6.0 and 35°C.

(a) *Titrimetric method.* The papain-catalysed hydrolyses of the methyl esters were followed by pH-stat as described by Lowe & Williams (1965).

(b) *Spectrophotometric method.* The papain-catalysed hydrolyses of the p-nitrophenyl esters and the anilides were followed spectrophotometrically as described by Lowe & Williams (1965), in McIlvaine's sodium phosphate–citrate buffer. The wavelengths chosen for the kinetic studies and the relevant extinction coefficients were as follows: p-nitrophenyl esters 340 nm (ϵ 0), p-nitrophenol (ϵ 6400); p-anisidides 315 nm (ϵ 0), p-anisidine (ϵ 560); p-nitroanilides 410 nm (ϵ 0), p-nitroaniline (ϵ 9000). Corrections were made for the non-enzymic hydrolysis of p-nitrophenyl esters. Non-enzymic hydrolysis of anilides was not detected under the conditions of the experiments.

Michaelis–Menten parameters, $k_{cat.}$ and K_m were determined from the initial velocities (less than 5% reaction) by a Lineweaver–Burk plot and the method of least squares. An English Electric KDF9 digital computer enabled the least-squares results to be calculated automatically from the kinetic results.

Lucas & Williams (1969) have shown that in 20% (v/v) acetonitrile–water there is little change in the Michaelis–Menten parameters for the papain-catalysed hydrolysis of methyl hippurate compared with water alone.

RESULTS AND DISCUSSION

The results shown in Table 1 reveal the importance of the amide bond adjacent to the carboxylic acid derivative undergoing hydrolysis. It is, however, significant that $k_{cat.}$ (which may reasonably be assumed to represent k_{+3}) for p-nitrophenyl

Table 1. *Michaelis–Menten parameters for the papain-catalysed hydrolysis of some* p-*nitrophenyl esters and* p-*anisidides at* pH 6.0 *and* 35°C

The $k_{cat.}$ values are related to a value of 2.7 s⁻¹ for methyl hippurate (Lowe & Williams, 1965).

Substrate	Organic solvent	$k_{cat.}$ (s⁻¹)	K_m (mM)	$k_{cat.}/K_m$ (M⁻¹·s⁻¹)	k_{-OH} (M⁻¹·s⁻¹)
C_6H_5-CO-NH-CH_2-CO-O-C_6H_4NO_2	1.9% CH_3CN	2.6±0.1	0.0117±0.0003	222000±13000	300†
C_6H_5-CO-CH_2-CH_2-CO-O-C_6H_4NO_2	10% (CH_3)_2N-CHO	2.4±0.5	0.53±0.12	4500±220	165
C_6H_5-CH_2-CH_2-CH_2-CO-O-C_6H_4NO_2	10% (CH_3)_2N-CHO	—	—	7	77
C_6H_5-CO-NH-CH_2-CO-NH-C_6H_4CH_3	20% (CH_3)_2N-CHO	0.024±0.006	15.9±4.3	1.5±0.1	
C_6H_5-CO-CH_2-CH_2-CO-NH-C_6H_4OCH_3	20% (CH_3)_2N-CHO	—	19.5*	—	
C_6H_5-CH_2-CH_2-CH_2-CO-NH-C_6H_4OCH_3	20% (CH_3)_2N-CHO	—	—	—	

* K_i determined by competitive inhibition of the hydrolysis of methyl hippurate.

† This is for C_6H_5-CH_2-O-CO-NH-CH_2-CO-O-C_6H_4NO_2 because the base-catalysed hydrolytic mechanism for p-nitrophenyl hippurate is anomalous (de Jersey, Willadsen & Zerner, 1969).

3-benzoylpropionate is essentially the same as that for p-nitrophenyl hippurate. This shows that the specificity of the enzyme is reflected entirely in K_m for these substrates. However, a better guide to the specificity of an enzyme is provided by $k_{cat.}/K_m$ since this truly represents k_{+2}/K_s (if $k_{-1} \gg k_{+2}$) even though K_m may incorporate non-productive binding constants (Bender & Kézdy, 1965). Replacement of the amino group of the amide bond by CH_2 lowers the overall rate of hydrolysis ($k_{cat.}/K_m$) about 50-fold and replacement of the carbonyl group by CH_2 lowers $k_{cat.}/K_m$ a further 640-fold. The overall effect of replacing the amide group by CH_2–CH_2 is to decrease the rate of hydrolysis ($k_{cat.}/K_m$) by a factor of more than 30000. By comparison the rate constants for the hydroxyl ion-catalysed hydrolysis of the same esters differ only by a factor of 4, clearly demonstrating the specificity of the enzyme for the peptide bond in the substrate adjacent to the site of cleavage.

For the papain-catalysed hydrolysis of the p-anisidide of hippuric acid $k_{cat.}$ is much smaller than for the p-nitrophenyl ester. On the basis of the three-step reaction pathway this must represent k_2, the rate constant for the acylation step and so K_m is the equilibrium binding constant K_s. The p-anisidide of 3-benzoylpropionic acid is not hydrolysed by papain at a measurable rate, but it does act as a competitive inhibitor of the papain-catalysed hydrolysis of methyl hippurate. The inhibition constant K_i for 3-benzoylpropionyl p-anisidide and the equilibrium binding constant K_s for hippuryl p-anisidide should be directly comparable and are seen to be identical within the statistical error (Table 1). This observation indicates that the additional binding energy of the hippuryl

moiety compared with the 3-benzoylpropionyl residue is used to facilitate catalysis. In particular it is used to facilitate the acylation step (k_{+2}), since no difference is observed in $k_{cat.}$ for the corresponding p-nitrophenyl esters which represents the deacylation rate constant k_{+3}. This is a striking example of kinetic specificity which is manifest in only one of the two catalytic steps. The ineffectiveness of the p-anisidide of 4-phenylbutyric acid as a competitive inhibitor provides evidence for the importance of the carbonyl group of the amide in binding.

The data presented in Table 2 provide evidence that the specificity of papain for the acyl residue on the N-terminus of the amino acid derivative undergoing cleavage is also reflected almost exclusively in the acylation step (k_{+2}). The similarity of $k_{cat.}$ for the methyl and p-nitrophenyl esters of hippuric acid and N-acetyl-L-phenylalanylglycine, indicate that this represents the deacylation rate constant, k_{+3}, for these substrates. It seems reasonable to assume that $k_{cat.}$ for the p-nitrophenyl ester of N-acetylglycine also represents k_{+3}. The specificity of the enzyme towards these substrates is clearly not manifest in the deacylation step, but in the binding or acylation as shown by the relative rate constants, $k_{cat.}/K_m$.

The observed catalytic constant ($k_{cat.}$) for the papain-catalysed hydrolysis of the p-nitroanilide of hippuric acid is just 1000 times smaller than that for the p-nitrophenyl ester and must represent therefore, the acylation step, k_{+2}. The observed catalytic constant ($k_{cat.}$) for the p-nitroanilide of N-acetyl-L-phenylalanylglycine is sufficiently close to $k_{cat.}$ for the p-nitrophenyl ester for it to be a composite constant of k_{+2} and k_{+3}. By using k_{+3} =

Table 2. *Michaelis–Menten parameters for the papain-catalysed hydrolysis of the* p-*nitroanilides and the methyl and* p-*nitrophenyl esters of some* N-*acylglycines at* pH 6.0 *and* 35°C

The values for N-acetylglycine methyl ester are taken from Lucas & Williams (1969) and converted to the same scale as the values for methyl hippurate from Lowe & Williams (1965).

Substrate	Organic solvent	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ (M$^{-1} \cdot$s^{-1})	Rel. $k_{cat.}/K_m$
CH$_3$-CO-Gly-NH-C$_6$H$_4$-NO$_2$	20% (CH$_3$)$_2$N-CHO	—	—	$8.7 \pm 1.0 \times 10^{-5}$	1
C$_6$H$_5$-CO-Gly-NH-C$_6$H$_4$-NO$_2$	20% (CH$_3$)$_2$N-CHO	$2.6 \pm 1.9 \times 10^{-3}$	7.2 ± 5.1	0.36 ± 0.03	4.15×10^3
CH$_3$-CO-Phe-Gly-NH-C$_6$H$_4$-NO$_2$	10% (CH$_3$)$_2$N-CHO	1.3 ± 0.2	0.88 ± 0.1	1500 ± 200	1.72×10^7
CH$_3$-CO-Gly-O-CH$_3$	10% CH$_3$CN	—	—	1.35	1
C$_6$H$_5$-CO-Gly-O-CH$_3$	—	2.7 ± 0.1	20.5 ± 2	133 ± 3	99
CH$_3$-CO-Phe-Gly-O-CH$_3$	—	5.4 ± 0.2	0.032 ± 0.001	$1.7 \pm 0.15 \times 10^5$	1.26×10^5
CH$_3$-CO-Gly-O-C$_6$H$_4$-NO$_2$	2% (CH$_3$)$_2$N-CHO	2.0 ± 0.1	0.68 ± 0.2	$3.0 \pm 0.2 \times 10^3$	1
C$_6$H$_5$-CO-Gly-O-C$_6$H$_4$-NO$_2$	1.9% CH$_3$CN	2.6 ± 0.1	$1.17 \pm 0.03 \times 10^{-2}$	$2.2 \pm 0.1 \times 10^5$	74
CH$_3$-CO-Phe-Gly-O-C$_6$H$_4$-NO$_2$	2% (CH$_3$)$_2$N-CHO	6.6 ± 0.1	$3.90 \pm 0.1 \times 10^{-4}$	$1.7 \pm 0.2 \times 10^7$	5.66×10^3

6.6s^{-1} (i.e. $k_{cat.}$ for the p-nitrophenyl ester), k_{+2} is found to be 1.6s^{-1} and $K_s = 1.1$mM. These calculated constants show that the specificity is very largely manifest in the acylation constant k_{+2}. The three sets of relative rate constants $k_{cat.}/K_m$ (Table 2) indicate that the enzyme shows greatest specificity towards the substrates that are most difficult to hydrolyse. This is clearly relevant to the hydrolysis of the peptide bond.

It is clear from the kinetic results that the specificity of papain is manifest in the acylation step for the substrates shown in Tables 1 and 2. The acyl-enzyme intermediate formed through the thiol group of cysteine-25 should therefore be essentially strain-free, whereas the enzyme–substrate complex must be distorted. It was decided therefore to see if these observations could lead to a plausible model of these two intermediates, by using the known tertiary structure of the enzyme. Model building began with the acyl-enzyme since in addition to being strain-free it had the further advantage of possessing a covalent link between the substrate and the enzyme. It seemed reasonable to assume that the peptide bond adjacent to the thiol ester would interact with the enzyme by hydrogen bonding. Since this type of bonding is highly directional and requires a precise geometry, it was readily seen that the peptide bond must bridge the cleft in the enzyme by forming hydrogen bonds with the NH group of glycine-66 and the C=O group (in the peptide backbone) of aspartic acid-158 (see Plate 1*b*). This type of binding is strikingly similar to the main binding in lysozyme in which the amide bond of an N-acetylglucosamine

residue bridges the cleft to form hydrogen bonds with a main chain C=O and NH group on each side (Blake *et al.* 1967). If the enzyme conformation remained unaltered it would appear that the N–H$\cdots$O bond distances for each of these hydrogen bonds would be 2.4–2.5 Å which is shorter than the minimum distance of 2.67 Å for this type of hydrogen bond (Pauling, 1960). This is an important point which will be returned to later, but it will be assumed for the moment that this is the correct mode of binding since no other appears to be available.

The amino acid derivative undergoing hydrolysis is now precisely orientated with respect to the enzyme, and it is clear why papain shows stereospecificity for L-amino acids. With the L-configuration the α-CH bond points into the body of the enzyme, and any group larger than H would give rise to severe interaction. The β-CH$_2$ group of an L-amino acid derivative (or CH$_3$ group in the case of L-alanine) would make good hydrophobic contact with the side chain of tryptophan-26. The γ-atom(s) would fit between the α-CH$_2$ groups of glycine-23 and glycine-65 and atoms further removed from the asymmetric carbon atom would probably be directed towards the solvent. It is noteworthy that a larger side chain than that of L-alanine could not be accommodated if residue 23 of the enzyme was anything other than glycine; in the closely related cysteine proteinases, ficin and stem-bromelain, a glycine residue is found in the same relative position, i.e. two residues towards the N-terminus of the active-centre cysteine residue (Husain & Lowe, 1970). The enzyme would also

prevent a β-branched side chain being accommodated at this site and so account for the inability of papain to cleave peptides at a valine residue even when it is flanked by L-phenylalanine at its N-terminus (Berger & Schechter, 1970).

The propensity for papain to cleave peptides at an amino acid residue adjacent to a phenylalanine or tyrosine residue has been recognized by Berger & Schechter (1970). However, it has been known for over 30 years that N-benzoyl, N-benzoyloxycarbonyl and L-leucyl residues are very effective at promoting cleavage by papain at the peptide bond of the adjacent amino acid residue (Bergmann, Zervas & Fruton, 1935, 1936). These observations strongly indicate that there must be a site that can bind hydrophobic residues. The precise geometry of the binding of the first peptide bond greatly restricts the possible sites for such an interaction. There is, however, a quite deep hydrophobic pocket in the cleft of the enzyme whose surface is made up mainly from the side chains of tyrosine-67, proline-68, tryptophan-69, phenylalanine-207, alanine-160, valine-133 and valine-157 (see Plate 1a). The side chains of a phenylalanine residue or tyrosine residue appear to be perfectly accommodated in this site making good hydrophobic contacts with the residues lining it (see Plate 1b). Two critical binding sites have therefore been defined for the amino acid residue of the substrate adjacent to that undergoing cleavage, namely the C=O group and the side chain, and it should now be possible to see why the enzyme shows stereospecificity for L-amino acid residues at this position. Inspection of the model shows that the α-CH of an L-amino acid at this site points into the body of the protein and there would appear to be insufficient space to accommodate the acylamino group of a D-amino acid. However, the stereospecificity of this site for L-alanine must be assured by further contact with the enzyme since its side chain cannot be expected to bind in the hydrophobic pocket. Inspection of the model shows that the NH group of a residue at this site is well placed for hydrogen bonding with the side-chain carboxylic acid group of aspartic acid-158. Thus stereospecificity is assured even though the side chain of the amino acid may not be capable of binding in the hydrophobic pocket. This binding also accounts for the fact that N-acetyl-L-phenylalanylglycine derivatives are considerably better substrates for papain than N-benzoyloxycarbonylglycine derivatives.

The results presented in Tables 1 and 2 indicate that the specificity of papain is largely manifest in the acylation step, k_{+2}, for these substrates. It follows therefore that the enzyme–substrate complex must be distorted to facilitate catalysis. Model building shows that the very precise geometry required for binding the peptide bond adjacent to the bond about to undergo cleavage will lead to distortion of the substrate and enzyme to relieve the otherwise severe non-bonded interaction between the bond about to undergo cleavage and the α-CH of histidine-159. From the model of the enzyme–substrate complex it would seem that this non-bonded interaction could be relieved by forcing the carbon atom of the amide or ester bond about to be cleaved toward the sp^3 hybridized configuration (see Plate 2a), and this could well be accompanied by rotation about the C–N bond. Both of these effects could of course contribute to an increase in the electrophilicity of the peptide bond and so facilitate attack by the thiol group. It may well be that this distortion is further facilitated by the peptide C=O group of aspartic acid-158 hydrogen-bonding with the NH of the leaving group, so forming a bifurcated hydrogen-bonding system with the peptide about to undergo cleavage and the adjacent peptide bond. However, movement of the imidazole group of histidine-159 and the thiol group of cysteine-25 seems to be excluded or be very slight because the pH-profiles for the acylation step (k_2) irrespective of the substrate specificity are very similar. However, more convincing evidence is provided by the striking observation that even saturating concentrations of the substrate N^{α}-benzoyl-L-arginine ethyl ester do not affect the rate constant for the irreversible inhibition of papain by chloroacetamide (Whitaker, 1969) or chloroacetic acid (Sluyterman, 1968). Moreover the pH-dependence for the inhibition of papain by chloroacetic acid is bell-shaped with apparent pK_a values similar to those found for the acylation step, k_{+2}, in substrate hydrolysis (Chaiken & Smith, 1969). These observations not only suggest that there is little or no movement of the catalytic groups between the enzyme and the enzyme–substrate complex but also indicate that the substrate does not hinder the approach of these reagents. N^{α}-Benzoyl-L-arginine ethyl ester does, however, hinder the activation of reversibly inactivated papain by cysteine (Sluyterman, 1966). All these observations are satisfactorily accounted for by the model. In the alkylation reactions the thiol group of cysteine-25 is the nucleophile and the halide ion of the reagent the leaving group, whereas in the activation mechanism the thiol of the activator nucleophilically attacks the disulphide bond and the thiol group of cysteine-25 is the leaving group (Sluyterman, 1967). The model shows that N^{α}-benzoyl-L-arginine ethyl ester would not hinder the approach of the alkylating reagents but would hinder the required approach of the activating reagent.

Evidence suggesting that it is the leaving group of the substrate that is in close contact with the enzyme and not the C=O group, is provided by the

EXPLANATION OF PLATES 1 AND 2

Anaglyphs of (1*a*) the proposed active site of papain, (1*b*) the acyl-enzyme, (2*a*) the enzyme–substrate complex, and (2*b*) the putative enzyme–substrate tetrahedral intermediate with L-phenylalanylalanine amide as substrate. C, N, O and S atoms are represented by circles of increasing size. Spectacles for viewing the anaglyphs are provided with this issue. The left eye should view through the red eyepiece and the right eye through the green eyepiece.
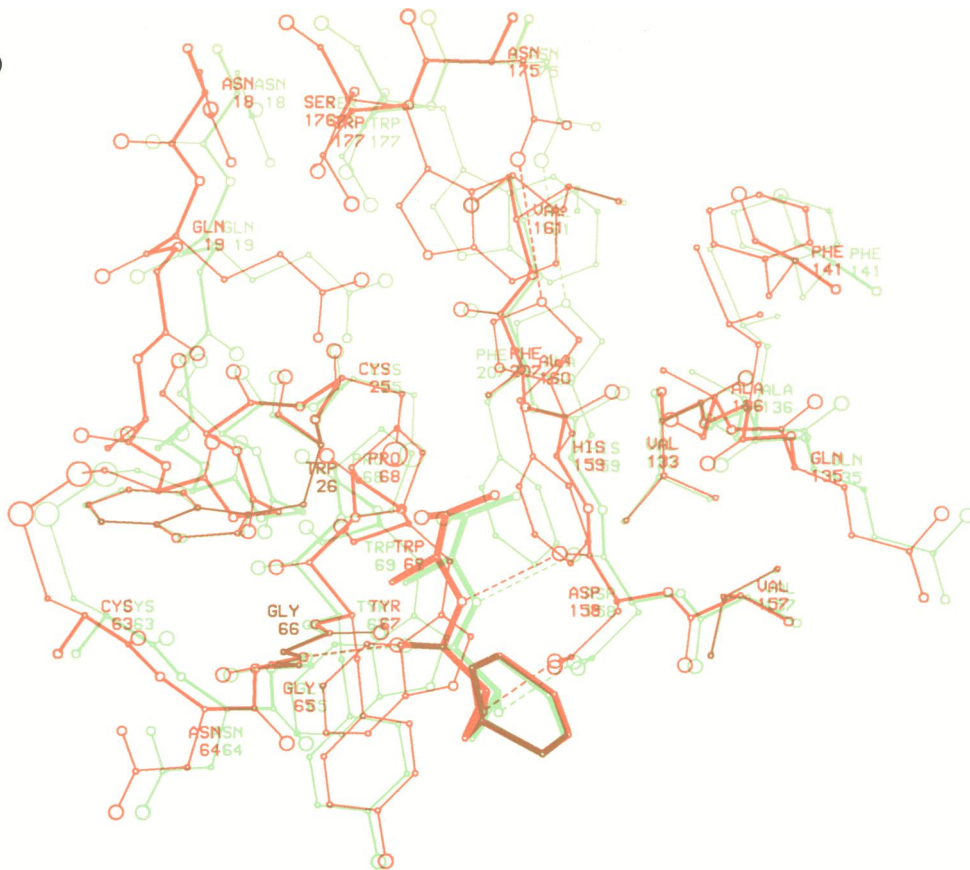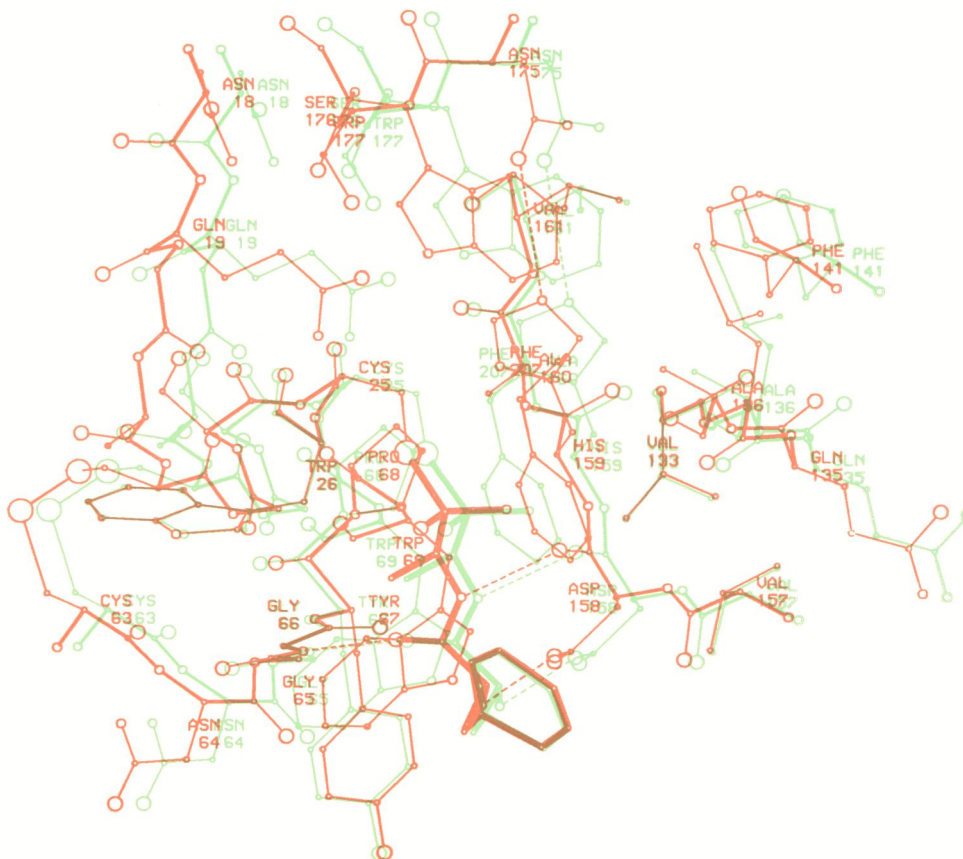
(1a)
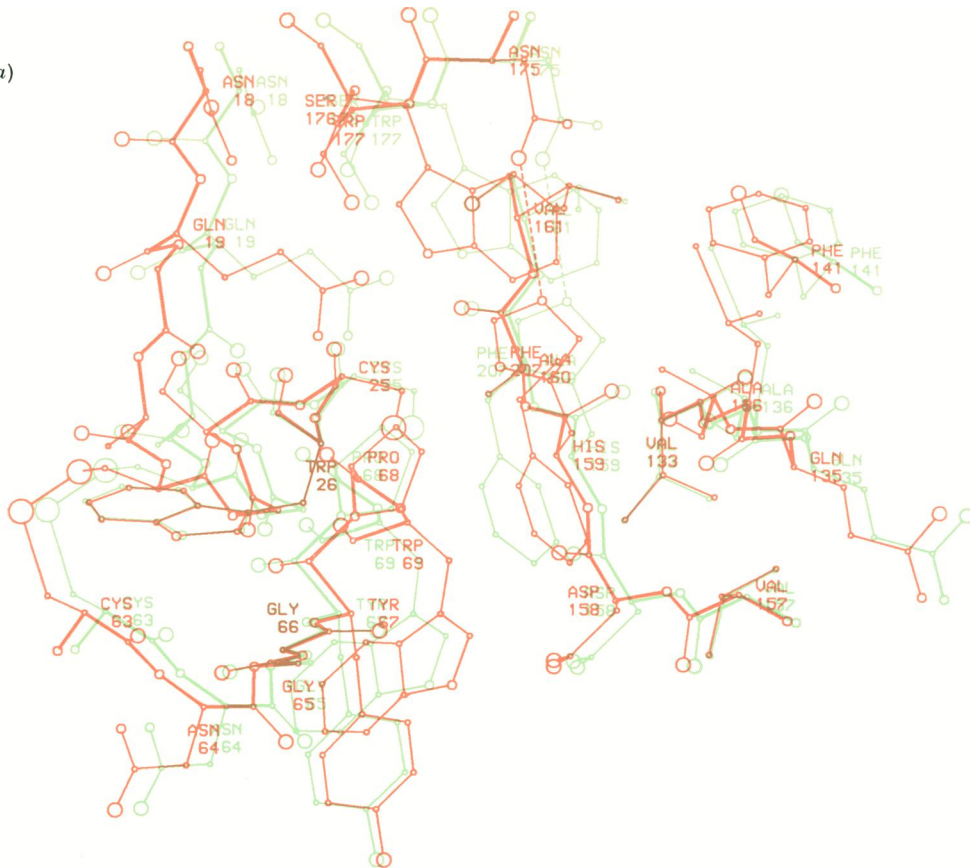
(1b)
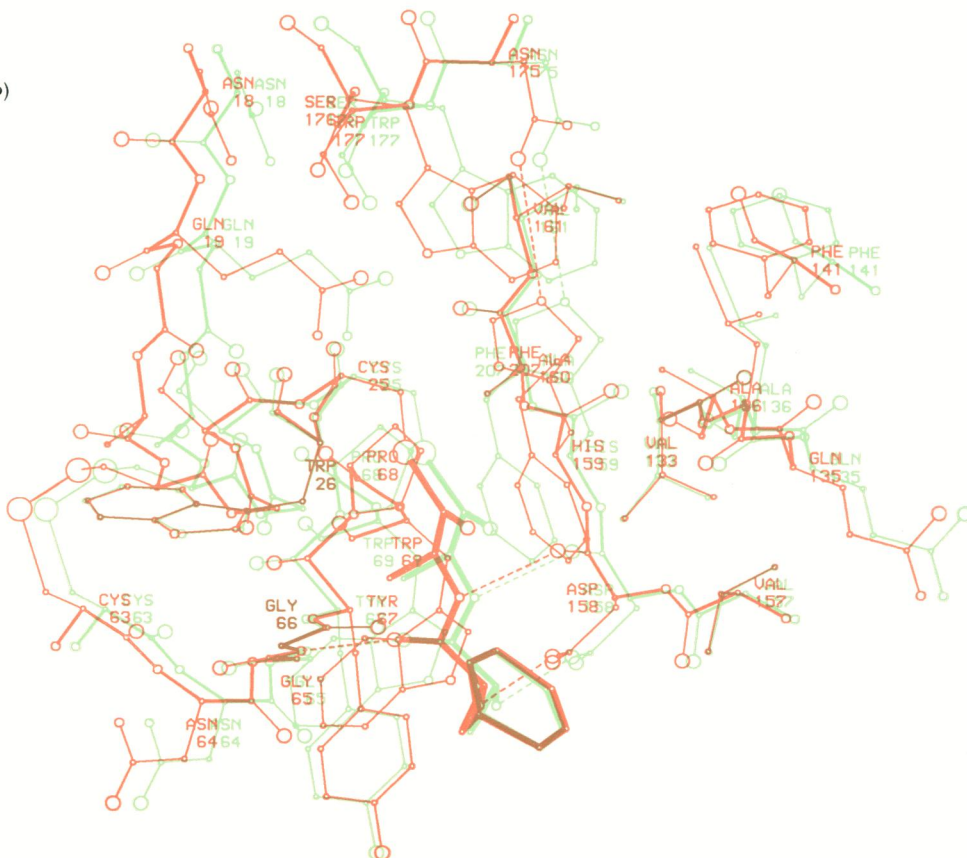
(2a)

(2b)

Table 3. *Competitive inhibition binding constants derived from the inhibition of the papain-catalysed hydrolysis of methyl hippurate at pH 6.0 and 35°C and in the presence of 2% acetonitrile*

The values in the second K_i column are taken from Lucas & Williams (1969) and are in 0.5% acetonitrile.

Inhibitor	K_i (mM)	K_i (mM)
CH_3–CO–NH–CH_2–CN	44*	40.8 ± 4.1
C_6H_5–CO–NH–CH_2–CN	0.46	0.382 ± 0.034
CH_3O–CO–Phe–NH–CH_2–CN	0.0018	
CH_3–CO–NH–CH_2–CSNH$_2$	30*	
C_6H_5–CO–NH–CH_2–CSNH$_2$	2.3*	
CH_3O–CO–Phe–NH–CH_2–CSNH$_2$	0.61	

* These values were determined by G. Allen and E. D. Sinclair.

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observation that papain does not cleave a peptide bond in which the leaving group is proline or sarcosine (*N*-methylglycine) (Bergmann *et al.* 1935). Such residues could be accommodated with the C=O group making close contact with the α-CH group of histidine-159 and the leaving group pointing towards the solvent, but in such an orientation the leaving group would be unable to accept a proton from the catalytic groups which it requires to leave. Moreover the enzyme would appear not to provide stereospecificity for the leaving group, which is known to obtain (Berger & Schechter, 1970, and references cited therein). However, more compelling evidence is provided by the fact that the binding of thiohippurylamide to papain (K_i 2.3mM; Table 3) is almost 100-fold better than hippurylamide (K_s 202mM; Lowe & Williams, 1965). Since the thiocarbonyl group has greater steric requirements than the carbonyl group, it seems likely that the amide (and thioamide) group is orientated so that the leaving group and not the carbonyl (or thiocarbonyl) group is in a sterically restricted environment.

The model of the enzyme–substrate complex (Plate 2a) also explains why papain is an endopeptidase. If the leaving group of the substrate were the terminal amino acid its carboxyl group would be only 3–4 Å from the carboxyl group of aspartic acid-158. At the optimum pH value for papain-catalysed hydrolysis these would both be ionized and electrostatically repel each other, so decreasing considerably the free energy of binding. The slow cleavage of glutamic acid residues is similarly explained.

Although there is no compelling evidence for the involvement of a tetrahedral intermediate between the enzyme–substrate complex and the acyl-enzyme in the reaction pathway, by analogy with simple ester and amide hydrolysis such a transient intermediate may well be involved. If such an inter-

mediate is involved the model shows (Plate 2b) that some but not all of the non-bonded interaction between the leaving group and the protein would be released. For substrates with the smaller acylation rate constants, the transition state will more closely resemble the tetrahedral intermediate (or acyl-enzyme) and so would be expected to gain most from kinetic specificity. This expectation is clearly evident from the relative $k_{cat.}/K_m$ results in Table 2.

The proposed model for the enzyme–substrate complex (Plate 2a) accounts for the observed kinetic specificity of papain. It follows from this model that the enzyme–product complex (EP_2) should be subject to similar distortion. This has the very considerable advantage that the product P_2 is not bound to the enzyme so tightly as would otherwise be the case, thereby ensuring that the product inhibition does not seriously impair enzymic activity. This conclusion suggests that the extensive data of Berger & Schechter (1970) on the product-inhibition constants of peptides would be a poor guide to the specificity of the enzyme, unless (as seems likely) they behave as virtual substrates and so inadvertently provide a valid indication of the enzyme specificity. The overall rates of hydrolysis of peptides should in general be a more reliable guide to the specificity of the enzyme.

The proposed model for the enzyme–substrate complex (Plate 2a) allows the prediction to be made that replacement of the amide or ester bond about to undergo cleavage by a group with less steric requirement should lead to a marked increase in binding for those inhibitors related to the more specific substrates.

The inhibition binding constants for three *N*-acylaminoacetonitriles and the corresponding thioamides are shown in Table 3. The nitrile group according to the model should have little or no non-bonded interaction with the enzyme, whereas the thioamide would have to be distorted to avoid severe interaction with the α-CH group of histidine-159. The much greater change in the binding constants of the nitriles compared with the thioamides provides good evidence to support the suggestion that distortion occurs in papain–substrate complexes and establishes that it is in the region of the peptide bond that is about to be cleaved that distortion occurs. The slightly better binding of *N*-acetylthioglycinamide compared with the corresponding nitrile suggests that when the binding energy is weak less distortion of the thioamide group is effected and its own binding contribution more than compensates for the loss of binding energy due to distortion.

The ratio of the inhibition binding constants for *N*-benzoylaminoacetonitrile and *N*-methoxycarbonyl-L-phenylalanylaminoacetonitrile is 255 and

the ratio of the acylation rate constants (k_{+2}) for the p-nitroanilides of N-benzoylglycine and N-acetyl-L-phenylalanylglycine is about 600. This factor of approx. 2 between these ratios is found again in the ratio of the deacylation rate constants, as seen in $k_{cat.}$ for the corresponding methyl (and p-nitrophenyl) esters (Table 2). A possible explanation of the similarity of these ratios is that when the acyl-L-phenylalanyl moiety is bound to the enzyme, the protein takes up a slightly different conformation from that when the hippuryl moiety is bound and so facilitates both acylation and deacylation by an additional factor of approx. 2. Whitaker (1969) observed that in the presence of high concentrations of N^α-benzoyl-D-arginine ethyl ester the rate of irreversible inhibition of papain by chloroacetamide was increased by a factor of 6. The model clearly indicates that an amino acid with the D-configuration could not be accommodated at the catalytic site but presumably the hydrophobic pocket could accept the benzoyl group and so produce a similar conformational change. Similarly Fink & Bender (1969) have shown that pentan-1-ol and propan-2-ol synergistically facilitate deacylation, presumably by binding in the hydrophobic pocket.

Comparison of the ratios of the inhibition binding constants, K_i, for the three acylaminoacetonitriles with the $k_{cat.}/K_m$ ratios for the corresponding p-nitroanilides, however, indicates that the overall effect of the N-acetyl-L-phenylalanyl moiety is rather greater than is indicated by comparison of the inhibition binding constants with $k_{cat.}$ for the p-nitroanilides. A mechanism is also required to explain how binding of the phenylalanine side chain in the hydrophobic pocket can facilitate acylation when binding of the peptide bond of the substrate is so precisely orientated.

As was stated earlier, if the NH of glycine-66 and the C=O of aspartic acid-158 remain in the same relative positions as found in the native enzyme the two hydrogen bonds formed would be short by perhaps 0.2–0.3 Å. (Although the accuracy of the atomic co-ordinates varies in different parts of the structure the atoms of the peptide backbone in general are determined most accurately. The cleft in the enzyme between the NH of glycine-66 and the backbone C=O of aspartic acid-158 appears to be about 0.6 Å narrower than the distance required to provide the two good hydrogen bonds. The error on this deficiency in distance must of course be comparatively large.) The model also shows that the side chain of the L-phenylalanine of the substrate, when bound in the hydrophobic pocket, forms close hydrophobic contacts with near neighbours of glycine-66 and aspartic acid-158 namely, tyrosine-67, proline-68 and tryptophan-69, in the glycine-66 chain, and valine-157 and alanine-

160 in the aspartic acid-158 chain; the NH group of the L-phenylalanine also forms a good hydrogen bond with the side chain of aspartic acid-158. It seems reasonable, therefore, that the effect of binding the N-acyl-L-phenylalanyl moiety is to open the cleft between the NH of glycine-66 and the C=O of aspartic acid-158, so that hydrogen bonding with the peptide bond of the substrate adjacent to that about to be cleaved, may make a greater contribution to the intrinsic binding energy. Bond compression is an energetically expensive process so that very small conformational changes effecting the distance between the NH of glycine-66 and the backbone C=O of aspartic acid-158 would be expected to influence considerably the intrinsic binding energy. Since only a fraction of the binding energy of the L-phenylalanine side chain could be expected to be relayed conformationally through the protein to the catalytic region, such a sensitive amplification mechanism for the specificity is required.

The model suggested for the enzyme–substrate complex (Plate 2a) implies a mutual distortion of the amide or ester bond about to be cleaved and the protein backbone in the region of histidine-159. It seems probable therefore that this distortion will slightly move the backbone C=O of aspartic acid-158 and so facilitate binding of the adjacent peptide bond of the substrate which in turn will facilitate distortion. Co-operativity of this kind is required to explain the greater ratios observed for the relative $k_{cat.}/K_m$ for the N-acylglycine p-nitroanilides (Table 2) compared with the inhibition constants for the N-acylaminoacetonitriles (Table 3).

The models proposed for the enzyme–substrate complex (Plate 2a) and the acyl-enzyme (Plate 1b) imply that at some stage between these intermediates there will be a conformational relaxation of the protein. Since the protein distortion is expected to be rather small this conformational relaxation is likely to be rapid and concomitant with the formation of the acyl-enzyme. However, with very reactive substrates it is conceivable that such a conformational relaxation could become a kinetically significant step.

REFERENCES

Bender, M. L. & Kézdy, F. J. (1965). *A. Rev. Biochem.* 34, 49.

Berger, A. & Schechter, I. (1970). *Phil. Trans. R. Soc. Ser.* B, **257**, 249.

Bergmann, M., Zervas, L. & Fruton, J. S. (1935). *J. biol. Chem.* **111**, 225.

Bergmann, M., Zervas, L. & Fruton, J. S. (1936). *J. biol. Chem.* **115**, 593.

Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C. & Sarma, V. R. (1967). *Proc. R. Soc. Ser.* B, **167**, 378.

Bryce, G. F., Roeske, R. W. & Gurd, F. R. N. (1965). *J. biol. Chem.* **240**, 3837.

Chaiken, I. M. & Smith, E. L. (1969). *J. biol. Chem.* **244**, 5095.

Curtius, T. & Goebel, F. (1888). *J. prakt. Chem.* **37** (2), 150.

de Jersey, J., Willadsen, P. & Zerner, B. (1969). *Biochemistry, Easton,* **8**, 1959.

Drenth, J., Jansonius, J. N., Koekoek, R., Swen, H. M. & Wolthers, B. G. (1968). *Nature, Lond.,* **218**, 929.

Fink, A. L. & Bender, M. L. (1969). *Biochemistry, Easton,* **8**, 5109.

Husain, S. S. & Lowe, G. (1970). *Biochem. J.* **117**, 341.

Jencks, W. P. (1969). *Catalysis in Chemistry and Enzymology,* p. 282. New York: McGraw–Hill Book Co.

Johansen, J. T. & Ottesen, M. (1968). *C. r. Trav. Lab. Carlsberg,* **36**, 265.

Johnson, T. B. & Burnham, G. (1912). *Am. chem. J.* **47**, 235.

Johnson, T. B. & Gatewood, E. (1929). *J. Am. chem. Soc.* **51**, 1815.

Kimmel, J. R. & Smith, E. L. (1954). *J. biol. Chem.* **207**, 515.

Klages, A. & Haack, O. (1903). *Ber. dt. chem. Ges.* **36**, 1646.

Knowles, J. R., Sharp, H. & Greenwell, P. (1969). *Biochem. J.* **113**, 343.

Lowe, G. (1970). *Phil. Trans. R. Soc. Ser.* B, **257**, 237.

Lowe, G. & Williams, A. (1965). *Biochem. J.* **96**, 199.

Lucas, E. C. & Williams, A. (1969). *Biochemistry, Easton,* **8**, 5125.

Pauling, L. (1960). *The Nature of the Chemical Bond,* 3rd ed., p. 498. Ithaca: Cornell University Press.

Sluyterman, L. A. Æ. (1966). *Biochim. biophys. Acta,* **113**, 577.

Sluyterman, L. A. Æ. (1967). *Biochim. biophys. Acta,* **139**, 430.

Sluyterman, L. A. Æ. (1968). *Biochim. biophys. Acta,* **151**, 178.

Soejima, M. & Shimura, K. (1961). *J. Biochem., Tokyo,* **49**, 260.

Somerville, L. F. & Allen, C. F. (1933). *Org. Synth.* **8**, 12.

Whitaker, J. R. (1969). *Biochemistry, Easton,* **8**, 4591.

Wolthers, B. G., Drenth, J., Jansonius, J. N., Koekoek, R. & Swen, H. M. (1970). *Proc. int. Symp. Structure–Function Relationships of Proteolytic Enzymes,* p. 272. Copenhagen: Munksgaard.