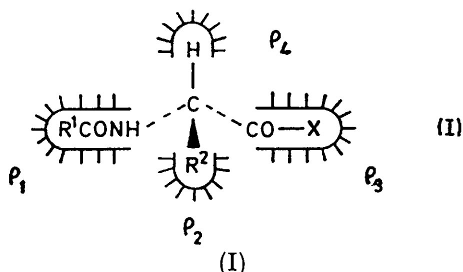
Proteolytic Enzymes. Nature of Binding Forces between Papain and its Substrates and Inhibitors

By **A. Williams,* E. C. Lucas, A. R. Rimmer,** and (Mrs.) **H. C. Hawkins,** University Chemical Laboratory, Canterbury, Kent

The binding of papain to its substrate $R^1CO{\cdot}NH{\cdot}CHR^2{\cdot}COX$ involves interaction between R^1CONH-, R^2-, and $-COX$ moieties and complementary sites (ρ_1, ρ_2 and ρ_3) on the enzyme. The ρ_1 and ρ_3 interactions involve lipophilic forces which are not of the charge-transfer type. The ρ_2 interaction is shown unequivocally not to involve electrostatic forces but depends on the length of the side-chain. Knowledge of the existence of the ρ_1 and ρ_3 binding sites has been used to design non-peptide reversible inhibitors of the enzyme.

PAPAIN catalyses the hydrolysis of substrates with general structure (I) [1] at the CO–X bond. Sub-sites ρ_{1-4} on the enzyme's active site interact with particular groups on the substrate (or inhibitor) to provide an overall binding force. Previous studies [2] have shown aromatic R^1 groups are more effective than methyl groups, suggesting that R^1 binds to its corresponding enzymic site with a lipophilic force.† The amide bond is also necessary for catalysis but can be replaced with no loss of efficiency by a sulphonamide group.[2a] The ρ_2 site was thought to be anionic [1] but this is not now a valid theory.[3] Studies with cinnamoyl-papain and various



(I)

† We use 'lipophilic force' as a blanket term to cover donor–acceptor (or charge-transfer) hydrophobic and van der Waals–London dispersion forces (see W. P. Jencks, 'Catalysis in Chemistry and Enzymology,' McGraw-Hill, New York, 1969, chs. 8 and 9).

[1] J. R. Kimmel and E. L. Smith, 'The Enzymes,' eds. P. D. Boyer, H. Lardy, and K. Myrback, Academic Press, 2nd edn., New York, 1960, **4**, 133.

[2] (*a*) E. C. Lucas and A. Williams, *Biochemistry*, 1969, **8**, 5125; (*b*) G. Lowe and E. C. Lucas, unpublished results quoted in G. Lowe, *Phil. Trans.*, 1970, *B***257**, 237.

[3] (*a*) W. Cohen and P. H. Petra, *Biochemistry*, 1967, **6**, 1047; (*b*) M. L. Bender and L. J. Brubacher, *J. Amer. Chem. Soc.*, 1966, **83**, 5880; (*c*) D. C. Williams and J. R. Whitaker, *Biochemistry*, 1967, **6**, 3711.

nucleophiles point to a lipophilic interaction with the ρ_3 site;[4] the ρ_4 site is probably a space accommodating only a hydrogen atom[5] but possessing no binding properties.

A study of the nature of the binding interactions requires a knowledge of equilibrium constants between enzyme and substrate or inhibitors. These can be derived from kinetic data assuming a three-step mechanism [equation (1)].[2a] The Michaelis–Menten rate equation derived from steady-state theory for this mechanism

$$\mathrm{ESH + RCOX} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ESH \cdots RCOX} \xrightarrow{k_2} \mathrm{ESCOR + HX} \quad (1)$$

$$\mathrm{ESCOR} \xrightarrow{k_3} \mathrm{RCO_2H + ESH}$$

has parameters (see results for definition of k_0 and K_m):

$$k_0 = k_2 \, . \, k_3/(k_2 + k_3) \quad (2)$$

$$k_0/K_m = k_2/K_s = k_1 \, . \, k_2/(k_{-1} + k_2) \quad (3)$$

The parameter K_s can be calculated if k_3 is known for those substrates where $k_2 < k_3$ and is a true equilibrium constant ($k_2 < k_{-1}$).[2a] Where $k_2 > k_3$, K_s cannot be calculated and the least complicated parameter involving it is k_0/K_m ($\equiv k_2/K_s$); this can be used as a measure of K_s in comparison with other values of k_0/K_m provided there is evidence that k_2 is invarient in the series. The effect of reversible inhibitors on catalysis is measured by an inhibition constant (K_i) which is a *bona-fide* equilibrium constant.

The magnitude of substrate or inhibitor binding constant with the enzyme can, in principle, be dissected into its microscopic contributions from ρ_1, ρ_2, ρ_3, and ρ_4 components. We have studied the effect of structure on binding to papain for the following inhibitors and substrates.

Inhibitors

(II) RCO·NH·CH₂·CN
R
a; *p*-NO₂·C₆H₄
b; *o*-NO₂·C₆H₄
c; 3,5-(NO₂)₂C₆H₃
d; PhCH₂O
e; 3-Pyridyl
f; N-methylpyridinium-3-yl I⁻
g; N-methylpyridinium-4-yl I⁻
h; Cyclohexyl
i; Me
j; Ph
(III) Cyclohexyl–NH·COPh
(IV) C₆H₅–CH₂·NH·SO₂–C₆H₄–Me
(V) 3-Pyridyl–CH₂·NH·COPh
(VI) PhCH₂·NH·CO–(3-pyridyl)
(VII) PhCH₂·NH·COPh
(VIII) PhCH₂·CH₂·CH₂·NH·COPh
(IX) 4-Pyridyl–CO·NH·NH₂

Substrates

(X) MeSO₂·NH·CH₂·CO·OMe
(XI) O₂N–C₆H₄–CO·NH·CH₂·CO·OMe
(XII) PhCH₂·O·CO·NH·CH(Me)CO·O–C₆H₄–NO₂ (*S*)
(XIII) PhCH₂·O·CO·NH(Me[CH₂]₃)CH·CO·O–C₆H₄–NO₂ (*S*)
(XIV) PhCH₂·O·CO·NH(C₇H₇SO₃⁻ N⁺H₃[CH₂]₄)CH·CO·OMe (*S*)
(XV) PhCH₂·O·CO·NH(C₇H₇SO₃⁻ N⁺H₃[CH₂]₄)CH·CO·O·CHMe₂ (*S*)
(XVI) PhCH₂·CO·NH·CH₂·CO·OMe
(XVII) Me–C₆H₄–SO₂·NH·CH₂·CO₂Me
(XVIII) Pyridinium(N-H)–CO·NH·CH₂·CO₂Me Cl⁻
(XIX) N-Methylpyridinium–CO·NH·CH₂·CO₂Et I⁻
(XX) MeSO₂·NH·CH₂·CO₂·CH₂–C₆H₄–NO₂
(XXI) PhCO·NH(PhCH₂)CH·CO₂Me (*S*)

Substrates (continued)

(XXII) MeCO·NH·CH₂·CO·O·CH₂–Cyclohexyl
(XXIII) Cyclohexyl–CO·NH·CH₂·CO·OMe
(XXIV) PhCO·NH·CH₂·CO₂·CH₂Ph
(XXV) PhCH₂·O·CO·NH(C₇H₇SO₃⁻ N⁺H[CH₂]₄)CH·CO·O·CH₂Ph (*S*)
(XXVI) MeCO·NH·CH₂·CO₂Me
(XXVII) MeSO₂·NH·CH₂·CO·O–C₆H₄–NO₂
(XXVIII) PhCH₂·O·CO·NH·CH₂·CO·O–C₆H₄–NO₂
(XXIX) MeSO₂·NH(PhCH₂)CH·CO·O–C₆H₄–NO₂ (*S*)
(XXX) PhC(:S)·NH·CH₂·CO·OMe
(XXXI) PhCO·NH·CH₂·CO·OMe

SCHEME 1

[4] (*a*) A. L. Fink and M. L. Bender, *Biochemistry*, 1969, **8**, 5109; (*b*) L. J. Brubacher and M. L. Bender, *J. Amer. Chem. Soc.*, 1966, **88**, 5871.

[5] J. de Jersey, *Biochemistry*, 1970, **9**, 1761.

EXPERIMENTAL

Materials.—Papain was prepared as described in the preceding paper.[6] Isopropyl *N*-α-benzyloxycarbonyl-L-lysine (XV) was prepared by refluxing the acid and a slight excess of toluene-*p*-sulphonic acid in isopropyl alcohol for five days. The solvent was evaporated under reduced pressure and the residual oil was crystallised twice from ethanol–ether to give a 60% yield of the tosyl salt. The methyl ester tosyl salt (XIV) was prepared in a similar fashion. Methyl mesylglycinate (X) was prepared by dichloromethane was added to the mixture which was then allowed to attain room temperature. After being set aside for 3 h the solution was extracted with water and the organic phase was dried and evaporated. Methanolic hydrogen chloride was used to obtain the hydrochloride. The methiodide (IIf) was prepared by refluxing the free nicotinyl base (IIe) in methanolic methyl iodide. Similar procedures gave the ethyl and methyl acylglycinates (XXIII), (XVIII), and (XIX) and the isonicotinyl derivatives (IIg). Other acylaminoacetonitriles (II) were

TABLE 1

Analytical and physical properties of substrates and inhibitors [a]

Compound	M.p. (t/°C)	Lit. m.p. (t/°C)	Found (%)			Formula	Calc (%)		
			C	H	N		C	H	N
(IIa)	142—142·5		52·8	3·7	20·0	$C_9H_7N_3O_3$	52·7	3·4	20·4
(IIb)	140—141		52·9	3·7	20·2	$C_9H_7N_3O_3$	52·7	3·4	20·4
(IIc)	190—191		43·0	2·9	22·2	$C_9H_8N_4O_5$	43·2	2·4	22·4
(IId)	59—61		63·1	5·3	14·7	$C_{10}H_{10}N_2O_2$	63·2	5·3	14·7
(IIe)	148—153		48·9	4·1	21·5	$C_8H_8ClN_3O$	48·6	4·1	21·3
(IIf)	152—153		35·5	3·4	14·0	$C_9H_{10}IN_3O$	35·6	3·3	13·8
(IIg)	204—206		35·5	3·2	13·6	$C_9H_{10}IN_3O$	35·6	3·3	13·8
(IIh)	101—103		64·7	8·3	17·1	$C_9H_{14}N_2O$	65·0	8·5	16·9
(III)	149—151	146—147 [b]							
(IV)	114—115	115—116 [c]							
(V)	179—182		62·7	5·0	11·1	$C_{13}H_{13}ClN_2O$	62·8	5·2	11·3
(VI)	72—74	72—73 [d]							
(VII)	100—101	105—106 [e]	79·9	6·3	6·7	$C_{14}H_{13}NO$	79·6	6·2	6·6
(VIII)	55—59	57—58 [e]							
(X)	29—32	32 [l]							
(XI)	148—151	155—156 [f]	50·6	4·2	11·8	$C_{10}H_{10}N_2O_5$	50·4	4·2	11·8
(XII)	78—79	78—97·5 [g]							
(XIII)	70—71·5		62·8	5·6	7·5	$C_{20}H_{22}N_2O_6$	62·1	5·7	7·3
(XIV)	82—84		56·7	6·3	6·0	$C_{22}H_{30}N_2O_7S$	56·7	6·4	6·0
(XV)	101—103		58·0	6·8	5·6	$C_{24}H_{34}N_2O_7S$	58·3	6·9	5·7
(XVI)	89—90	89—90 [h]							
(XVII)	92—93	92—93 [i]							
(XVIII)	140 (decomp.)		47·0	4·9	11·6	$C_9H_{11}ClN_2O_3$	47·0	4·8	12·1
(XIX)	142—145		37·8	3·8	7·8	$C_{11}H_{15}IN_2O$	37·7	4·3	8·0
(XX)	143·5—145		42·0	4·4	9·8	$C_{10}H_{12}N_2O_6S$	41·7	4·2	9·7
(XXI)	85—86	83·6—84·6 [j]							
(XXII)	B.p. 144—146 (0·005 mm)		60·6	8·8	6·8	$C_{11}H_{19}NO_3$	60·3	8·6	7·0
(XXIII)	96—99		60·0	9·0	6·9	$C_{10}H_{17}NO_3$	60·3	8·6	7·0
(XXIV)	87—89	87—89 [k]							

[a] Analyses by Mrs. M. J. Clark on a Hewlett–Packard 185 Analyser. Melting points were determined on a Kofler 'Thermospan' instrument. [b] G. Alliger, G. E. P. Smith, E. L. Carr, and H. P. Stevens, *J. Org. Chem.*, 1949, **14**, 962. [c] S. Wawzonek and D. Meyer, *J. Amer. Chem. Soc.*, 1954, **76**, 2918. [d] J. H. Billman and J. L. Rendall, *J. Amer. Chem. Soc.*, 1944, **66**, 540. [e] Dictionary of Organic Compounds, Eyre and Spottiswoode and Spon, London, 1965. [f] K. Miyatake and S. Kaga, *J. Pharm. Soc.* (*Japan*), 1952, **72**, 627. [g] M. Goodman and K. C. Stueben, *J. Amer. Chem. Soc.*, 1959, **81**, 3980. [h] S. Ose, Y. Yoshimura, I. Matsumoto, S. Moriguchi, and T. Usui, *J. Pharm. Soc. Japan*, 1950, **70**, 704. [i] E. Kaiser and E. P. Gunther, *J. Amer. Chem. Soc.*, 1956, **78**, 3841. [j] M. Goodman and L. Levine, *J. Amer. Chem. Soc.*, 1964, **86**, 2918. [k] J. C. Sheehan and E. J. Corey, *J. Amer. Chem. Soc.*, 1952, **74**, 4555. [l] P. T. Frangopol, A. T. Balaban, L. Baraladeaunu, and E. Gioranescu, *Tetrahedron*, 1961, **16**, 59.

treating mesyl chloride with methyl glycinate hydrochloride in chloroform in the presence of two molar proportions of triethylamine. The esters (XI), (XVI), and (XVII) were prepared in the same way from the corresponding acid chloride and methyl glycinate hydrochloride. 4-Nitrophenyl esters of *N*-α-benzyloxycarbonyl-L-alanine (XII) and *N*-α-benzyloxycarbonyl-L-norleucine (XIII) were prepared from the acid and 4-nitrophenol using dicyclohexylcarbodi-imide. *N*-Benzyloxycarbonyl aminoacetonitrile (IId) was prepared by treating benzyl chloroformate with aminoacetonitrile hydrogen sulphate in an excess of aqueous sodium hydroxide. Nicotinylaminoacetonitrile (IIa) was prepared by cooling a mixture of nicotinic acid (1 equiv.) and triethylamine (1 equiv.) in dichloromethane to −5°. Ethyl chloroformate (1 equiv.) was then added to form the mixed anhydride and the mixture was stirred for 30 min at −5°. Aminoacetonitrile hydrochloride (1 equiv.) and triethylamine (2 equiv.) in prepared from the acid chloride and aminoacetonitrile hydrogen sulphate using pyridine as solvent. 4-Nitrobenzyl mesylglycinate (XX) was prepared from the acid chloride and the alcohol in dichloromethane solvent at 5° with an equivalent proportion of pyridine. The amides (III)—(VIII) were prepared by Schotten–Baumann acylation of the corresponding amines and isonicotinohydrazide (IX) was purchased from Koch–Light Ltd., (m.p. 162—163°, lit.,[7] m.p. 163°). Cyclohexylmethyl *N*-acetylglycinate (XXII) and benzyl hippurate (XXIV) were prepared by acetylation and benzoylation respectively of cyclohexylmethylglycinate and benzyl glycinate hydrochlorides. Methyl *N*-α-benzoyl-L-phenylalaninate (XXI) was synthesised from methyl L-phenylalaninate hydrochloride and benzoyl chloride. Methyl thiobenzoylglycinate (XXX)

[6] A. Williams, E. C. Lucas, and A. R. Rimmer, preceding paper.
[7] H. Meyer and M. Mally, *Monatsh.*, 1912, **33**, 400.

was prepared from methylglycine and methyl dithiobenzoate. Methyl acetylglycinate (XXVI), 4-nitrophenyl mesylglycinate (XXVII), 4-nitrophenyl N-α-mesyl-L-phenylalaninate (XXIX), and benzyl N-α-benzyloxycarbonyl-L-lysine (XXV) were from other investigations.[2a,6,8] 4-Nitrophenyl N-α-benzyloxycarbonylglycine was bought from The Sigma Chemical Company.

Structures were confirmed by analysis (Table 1) and by i.r. and n.m.r. spectroscopy.

Acetonitrile was purified as described previously[6] and deionised water was used throughout. Buffer materials were of analytical reagent grade.

Methods.—Kinetic and computational methods are described elsewhere.[2a,6]

RESULTS

Hydrolysis of all the ester substrates obeyed Michaelis–Menten kinetics [equation (4)] and the results are recorded

$$\text{rate} = k_0[\text{E}][\text{S}]/([\text{S}] + K_m) \quad (4)$$

in Table 2 and illustrated in Figure 1. Where comparable the parameters are in good agreement with literature values [(XXVII), (XII), (XXV), and (XIV)].[3b,5,9] Values of k_0 for the benzyl and 4-nitrophenyl N-α-benzyloxycarbonyl-L-lysinates were sufficiently similar to be identified as k_3; using values of k_3 (= k_0) over a pH-range from a study of the benzyl ester[6] k_2 and K_s were calculated for the

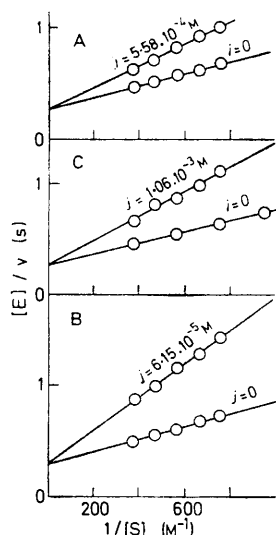


FIGURE 1 Typical kinetic data for acylaminoacetonitrile inhibitors using methyl hippurate as substrate. Lines are theoretical: A, benzyloxycarbonyl (IId); B, 2-nitrobenzoyl (IIb); C, 3-(N-methylpyridyl) iodide (IIf). See Table 4 for conditions

isopropyl ester (XV) using equations (2) and (3) and k_0 and K_m. Kinetic data are illustrated in Figure 2. The pH-profile of k_2 was bell-shaped (pK_{a1}, 4·55; pK_{a2}, 8·38) in agreement with those for other substrates[2a] and K_s pH-independent (Table 3 and Figure 3).

The inhibitors were reversible and obeyed a competitive rate law [equation (5)]; results are recorded in Table 4.

$$\text{rate} = k_0[\text{E}][\text{S}]/\{[\text{S}] + K_m([I]/K_1 + 1)\} \quad (5)$$

Table 5 records values of k_2 and K_s derived using equations (2) and (3) and values of k_0 and K_m for substrates where $k_2 > k_3$. Values of k_3 were derived from k_0 for 4-nitrophenyl esters; this is a reasonable assumption because k_2 is expected to exceed k_3 owing to the good leaving ability of the 4-nitrophenyl group. In the series of esters already studied (hippurates,[9] acetylglycinates,[2a] mesylglycinates,[6] benzyloxycarbonylglycine[10]) a constant k_0 is observed for good leaving groups; thus by equation (2) $k_0 = k_3$ for the 4-nitrophenyl ester.

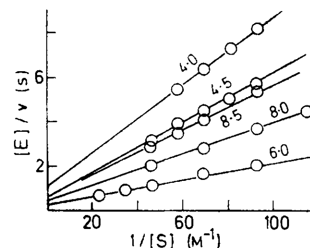


FIGURE 2 Typical kinetic data for isopropyl N-α-benzyloxycarbonyl-L-lysine (XV). Lines are theoretical; see Table 3 for conditions

TABLE 2

Michaelis–Menten parameters for some substrates of papain [a]

Substrate	k_0/s^{-1}	K_m/M ($\times 10^3$)	(k_0/K_m)/ l mol^{-1} s^{-1}
(XXVI) [b]	1·42 ± 0·9	632 ± 62	2·25
(XXVIII) [b]	7·87 ± 0·05 [d]		
(XII) [b]	18·2 ± 0·1 [d]		35 . 10^5 [e]
(XIII) [b]	34·2 ± 0·6	9·76 ± 0·04	35 . 10^5
(XXV) [c]	38·3 ± 0·5 [d]		
(XIV) [c]	20·7 ± 0·9	31·3 ± 3·4	661
(X) [c]	3·34 ± 0·13	176 ± 12	19·0
(XXVII) [c,f]	11·1 ± 0·9	1·93 ± 0·2	5·75 . 10^3
(XXIX)			39·8 . 10^3 [e]
(XVIII)	4·25 ± 0·14	43·0 ± 2·9	99
(XI)	4·02 ± 0·27	9·3 ± 1·0	432
(XIX)	0·28 ± 0·03	43·1 ± 0·66	6·5
(XVI)	3·2 ± 0·03	48·3 ± 3	66·7
(XVII)	0·94 ± 0·08	10·0 ± 2	94
(XXIII)	2·05 ± 0·1	3·97 ± 0·5	517
(XXII)	1·37 ± 0·2	11·6 ± 2	118
(XX)			1·99 . 10^3
(XXIV)	3·04 ± 0·07	0·552 ± 0·035	5·5 . 10^3 [g]
(XXX)	0·32 ± 0·01	18·5 ± 2	17·3
(XXI)	1·22 ± 0·05	2·35 ± 0·09	520

[a] pH 6·00, 35°. [b] 0·3M-Ionic strength. [c] 0·1M-Ionic strength. [d] Determined at [S] > K_m (pseudo-zero-order kinetics). [e] Determined at [S] < K_m (pseudo-first-order kinetics). [f] Ref. 6. [g] 20% (v/v) CH_3CN.

TABLE 3

Kinetic parameters for isopropyl N-benzyloxycarbonyl-L-lysine (XV) [a]

pH	k_0/s^{-1}	K_m/M ($\times 10^3$)	k_2/s^{-1}	K_s/M ($\times 10^3$)
4·0	1·09 ± 0·12	84·5 ± 11·4	1·15 ± 0·15	89 ± 14
4·5	2·25 ± 0·09	104 ± 5	2·47 ± 0·11	115 ± 8
5·0	3·25 ± 0·2	94·3 ± 7·7	3·69 ± 0·26	107 ± 13
6·0	4·09 ± 0·14	90·9 ± 3·9	4·8 ± 0·19	107 ± 7
7·0	4·2 ± 0·21	92·8 ± 5·8	4·95 ± 0·29	109 ± 11
8·0	3·34 ± 0·42	94·7 ± 14·7	3·8 ± 0·54	108 ± 2·7
8·5	2·04 ± 0·2	105 ± 12	2·2 ± 0·23	115 ± 21

[a] 25°, 0·3M-Ionic strength; initial substrate concentration 8—50 . 10^{-3}M; enzyme concentration *ca*. 10^{-5}M; results below pH 5 corrected for association of the product acid using pK_a = 3·53.

[8] A. Williams, *Biochemistry*, 1970, **9**, 3383.
[9] G. Lowe and A. Williams, *Biochem. J.*, 1965, **96**, 199.
[10] J. F. Kirsch and M. Igelström, *Biochemistry*, 1966, **5**, 783.

TABLE 4
Competitive inhibitors of papain [a]

Inhibitor	K_i/M ($\times 10^3$)	$K_i/(K_s/k_2)$ Methyl N-acylglycine	Methyl N-acyl-L-arginine
(IIi)	40·8 [d]	0·092	
(IId)	0·664 ± 0·05		
(IIa)	0·21 ± 0·03	0·091	1·0
(IIj)	0·38 [d]	0·093	0·73
(IIb)	0·038 ± 0·002		0·83
(IIc)	0·62 ± 0·068		0·94
(IIe)	0·784 ± 0·02	0·077	
(IIf)	10·0 ± 0·08	0·065 [c]	
(IIg)	27·0 ± 1·3		
(IIh)	0·0952 ± 0·004		
(III)	1·8 ± 0·3		
(IV)	40 ± 3·6		
(V)	90·5 ± 16		
(VI)	71·5 ± 7·2		
(VII)	15·2 ± 0·3		
(VIII)	>25 [e]		
(IX)	25·4 ± 0·6		

[a] Methyl hippurate used as standard substrate, pH 6·0, 35°, 0·3M-ionic strength. [b] Data for methyl esters from C. E. MacDonald and A. K. Balls, *J. Biol. Chem.*, 1957, **229**, 73. [c] Ratio for ethyl ester. [d] Ref. 2*a*. [e] Solubility difficulties precluded concentration of inhibitor greater than 0·025M.

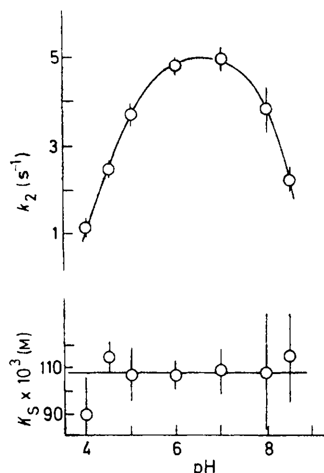


FIGURE 3 pH-Dependence for K_s and k_2 for isopropyl N-α-benzyloxycarbonyl-L-lysine (XV). Data from Table 3; lines are theoretical

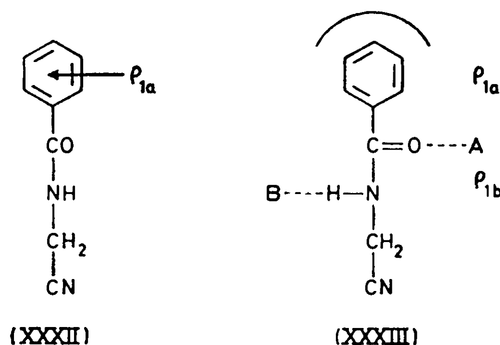
TABLE 5
Kinetic parameters for some ester substrates of papain [a]

Ester	k_2/s^{-1} [h]	K_s/M [h] ($\times 10^3$)	(k_2/K_s)/ l mol^{-1} s^{-1} [h]
Isopropyl hippurate	1·34	47	
Isopropyl mesylglycinate	1·11	233	
4-Nitrophenyl acetylglycinate			2390
Benzyl acetylglycinate			207 [b,e]
(XXVI)	3·0 [c,d]	1340	2·25
(X)	4·45 [c,d]	234	19
4-Nitrophenyl N-α-benzyloxycarbonyl-L-lysine	860 [g]	0·033 [g]	2·6 . 10^7 [g]
(XXV)	1000 [f]	1·1 [f]	9 . 10^5 [f]
(XIV)	81 [c]	120	662
(XV)	5·20 [c]	108	48·1
(XXXI)			246 [b]

[a] Values for lysine derivatives at 25°, the rest at 35°. [b] An error is noted for this value in Table 6 of ref. 2*a*. [c] This work. [d] $k_3 = k_0$ for the 4-nitrophenyl ester.[2a] [e] Ref. 2*a*. [f] Ref. 6. [g] Ref. 3*b*. [h] Limiting values.

DISCUSSION

ρ_1 *Site*.—The overall binding force between chymotrypsin and a specific inhibitor or substrate probably involves a number of mutually exclusive components.[11] While it may be that in papain the binding components are not mutually exclusive it is still useful to discuss the individual binding sites. Comparison of K_i, K_s, and k_2/K_s values for inhibitors and substrates varying only in the substituent on the α-nitrogen (Tables 2, 4, and 5) indicates that binding strength varies in the order: ArCO ~ $PhCH_2O{\cdot}CO$ > $MeSO_2$ > Ac. This order can be explained by the existence of two subsites: ρ_{1a} binding the aryl portion and ρ_{1b} binding the amide. Methyl nicotinylglycine (XVIII) reacts *more* efficiently



than its methiodide (XIX), nicotinylaminoacetonitrile (IIe) binds better than its methiodide (IIf), and k_2/K_s and K_i values for methyl cyclohexanoylglycine (XXIII) and cyclohexanoylaminoacetonitrile (IIh) indicate better binding than their corresponding aromatic analogues (IIj) and (XXXI). These results are not consistent with charge-transfer binding (XXXII); the inhibition constant for substituted benzoylaminoacetonitriles decreases with decreasing pK_a of the corresponding benzoic acid (Table 4, Figure 4) in accord with an acid–

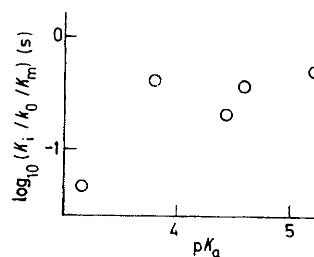


FIGURE 4 Dependence of K_i on pK_a for substituted benzoylaminoacetonitriles. See G. Kortum, W. Vogel, and K. Andrussow, 'Dissociation Constants of Organic Acids in Aqueous Solution,' Butterworths, London, 1961

base type binding (XXXIII). *X*-Ray crystallographic evidence has been advanced for the binding of the amide group of a substrate to the corresponding ρ_1 site in chymotrypsin involving hydrogen bonding with a base

[11] (*a*) M. L. Bender and F. J. Kézdy, *Ann. Rev. Biochem.*, 1965, **34**, 49; (*b*) G. E. Hein and C. Niemann, *J. Amer. Chem. Soc.*, 1962, **84**, 4494; (*c*) C. L. Hamilton, C. Niemann, and G. S. Hammond, *Proc. Nat. Acad. Sci. U.S.A.*, 1966, **55**, 664.

to the nitrogen NH.[12] It could be that the hydrogen bond from A to the amide oxygen is not so important as there is a fairly marked dependence of K_i on pK_a

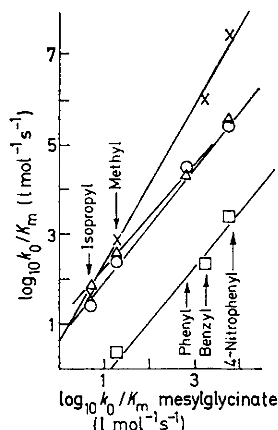


FIGURE 5 Dependence of k_2/K_s for esters of acylamino-acids on k_2/K_s for the corresponding mesylglycinate esters. ×, N-α-benzyloxycarbonyl-L-lysine; ○, hippurates; □, acetylglycinates; △, N-α-benzyloxycarbonylglycine

and because the K_m (Table 2) for methyl thiobenzoylglycinate (XXX) is not very different from that for the oxygen analogue (XXXI).[2a]

The intramolecular acylation rate constant k_2 is probably constant for methyl esters of N-α-acyl-L-argininates and for methyl esters of acylglycinates because the ratio of K_i of the acylaminoacetonitrile to K_s/k_2 is constant (Table 4). The constancy of k_2 for leaving groups is reflected in the identical (unit) slopes of the plot of $\log_{10} k_2/K_s$ for esters of acylglycinates *versus* $\log_{10} k_2/K_s$ for esters of mesylglycine (Figure 5). The k_2/K_s for esters of N-α-benzyloxycarbonyl-L-lysine is more sensitive to leaving group than is that for acylglycinates thus k_2 for a given leaving group does not, in general, equal that for an acylglycine ester. This can be attributed to the breakdown of the mutual exclusiveness of the ρ_1 and ρ_2 interactions.

ρ_2 *Site.* Substrates with cationic side-chains such as arginine or lysine derivatives are hydrolysed in the presence of papain faster than those with uncharged side-chains and it was thought [1] that the cation was attracted to an anionic site on the enzyme. Later work [3] showed that destruction of the cationic moiety by, for example, formylation did not alter the catalytic parameters. We find that the substrate isopropyl N-α-benzyloxycarbonyl-L-lysine (XV), which has a cationic side-chain, has a pH-independent K_s value in the pH-range 4—8·5 directly indicating the absence of a carboxylate ion interaction on the enzyme with the ammonium moiety of the lysine side-chain (Figure 3). As side-chain length is increased (Figure 6) the values of k_3 approach a maximal value.

ρ_3 *Site.*—Benzyl ester substrates of papain have k_2/K_s values closer to those for aryl than alkyl esters and aryl esters have k_2/K_s values some 100—1000-fold larger than alkyl esters. The difference can be accounted for, in part, by enhanced leaving-group ability (*i.e.* effect on k_2) but benzyl esters have reactivity to nucleophiles intermediate between that for methyl and ethyl esters.[13] These results can be explained by a lipophilic component (ρ_{3a}) of the ρ_3 site; the lipophilic interaction is probably not of the charge-transfer type because 4-nitrobenzyl (XX) and benzyl mesylglycinates [2a] have identical values for k_2/K_s and cyclohexylmethyl acetylglycinate (XXII) is *more* reactive than the benzyl ester.[2a] Kirsch [10] argued for the absence of leaving group binding with enzyme but Lowe and Bender [3b,9] found K_s varied for different leaving groups. Reaction of nucleophiles with cinnamoyl-papain revealed the existence of a lipophilic binding site for the nucleophile [4] which in the acylation reaction would be the leaving group.

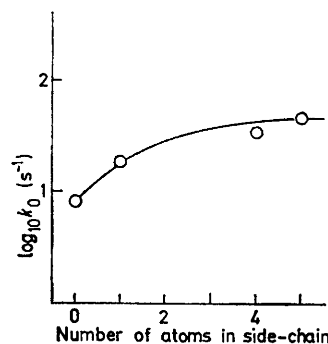
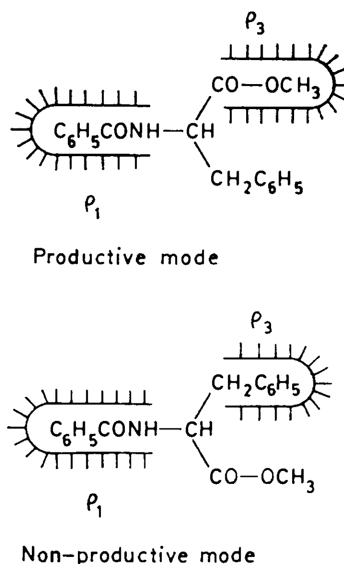


FIGURE 6 Effect of length of side chain on k_3 (k_0 for 4-nitrophenyl ester) for N-α-benzyloxycarbonyl-L-amino-acids

The possible existence of lipophilic binding sites at the active centre of papain prompted the synthesis of



potential linear inhibitors with lipophilic end groups (III)—(IX). Results in Table 4 indicate that as chain

[12] T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, 1969, **46**, 337.

[13] J. R. Robinson and L. M. Matheson, *J. Org. Chem.*, 1969, **34**, 1363.

length is increased inhibition falls off markedly. By comparison, simple compounds with only one lipophile such as 2-benzamidoethanol or benzyl alcohol [2a] have no observable inhibitory power. Isonicotinyl hydrazide was a comparatively good inhibitor and was studied because it is known to be an inhibitor of fibrinase a thiol protease in the blood coagulation system.[14]

Methyl N-α-benzoyl-L-phenylalaninate (XXI) has a lower K_m value than methyl hippurate in accord with the action of two lipophilic sites as opposed to only one, but its k_0 value is less than half. These results can be rationalised by the existence of a complex where a significant proportion of the substrate is bound in a non-productive [11b,c,15] way at the active centre.

[1/1413 *Received*, 10*th August*, 1971]

[14] (*a*) L. Lorand, A. Jacobsen, and J. Bruner-Lorand, *J. Clin. Invest.*, 1968, **47**, 268; (*b*) L. Lorand, R. F. Doolittle, K. Konishi, and S. K. Riggs, *Arch. Biochem. Biophys.*, 1963, **102**, 171; (*c*) J. Bruner-Lorand, T. Uryama, and L. Lorand, *Biochem. Biophys. Res. Commun.*, 1966, **23**, 828.

[15] (*a*) S. A. Bernhard and H. Gutfreund, *Proc. Internat. Symp. Enzyme Chem. Tokyo*, 1958, 124; (*b*) S. A. Bernard, *J. Cellular Comp. Physiol.*, 1959, **54** (suppl. 1), 256; (*c*) T. Spencer and J. W. Sturtevant, *J. Amer. Chem. Soc.*, 1959, **81**, 1874; (*d*) C. Niemann, *Science*, 1964, **143**, 1287.