Mechanism of Action and Specificity of Proteolytic Enzymes

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The mechanism of action of proteolytic enzymes (proteases) has been investigated intensively during the past decade in the absence of any detailed knowledge of enzyme structure.[1,2] This study is now coming to fruition because tertiary structures of proteins are rapidly becoming available.[3–7] The main reason for such intense application is that these enzymes are structurally the simplest, and details of their mechanism could act as a guideline in the study of more complex systems.

Proteases have a simple and necessary function in nature; they hydrolyse protein food into dialysable fragments in the digestive tracts of mammals. In plants they catalyse the turnover of protein and may form part of a defence mechanism in tropical species. Proteases of some bacteria destroy the reactive lactam bond in penicillin, whilst other bacterial proteases have a function similar to that of mammalian digestive enzymes.

The proteolytic enzymes discussed here have been chosen because they seem to represent a family of less well documented enzymes and because their chemistry is of considerable topical interest.*

1 General Properties

The reader should consult more general texts for a background to enzymology and protein chemistry.[9–13] Proteases catalyse the reaction (1), and although this

* Owing to the existence of several excellent reviews, α-chymotrypsin is only mentioned in passing.[1,8,9]

[1] M. L. Bender and F. J. Kézdy, *Ann. Rev. Biochem.*, 1965, **34**, 49.
[2] W. P. Jencks, *Ann. Rev. Biochem.*, 1963, **32**, 648.
[3] C. C. F. Blake, D. F. Koenig, G. A. Mair, A. C. T. North, C. D. Phillips, and V. R. Sarma, *Nature*, 1965, **206**, 757.
[4] G. Kartha, J. Bello, and D. Harker, *Nature*, 1967, **213**, 862.
[5] B. W. Matthews, P. B. Sigler, R. Henderson, and D. M. Blow, *Nature*, 1967, **214**, 652.
[6] G. N. Reeke, J. A. Hartsuck, M. L. Ludwig, F. A. Quiocho, T. A. Steitz, and W. N. Lipscomb, *Proc. Nat. Acad. Sci. U.S.A.*, 1967, **58**, 2220.
[7] H. W. Wyckoff, K. D. Hardman, N. M. Allewell, T. Inagami, L. N. Johnson, and F. M. Richards, *J. Biol. Chem.*, 1967, **242**, 3984.
[8] T. C. Bruice and S. J. Benkovic, 'Bio-organic Mechanisms', vols. 1 and 2, Benjamin, New York, 1966.
[9] S. G. Waley, *Quart. Rev.*, 1967, **21**, 379.
[10] M. Dixon and E. C. Webb, 'Enzymes', Longmans, London, 1964.
[11] H. Gutfreund, 'An Introduction to the Study of Enzymes', Blackwell, Oxford, 1965.
[12] K. J. Laidler, 'The Chemical Kinetics of Enzyme Action', Clarendon Press, Oxford, 1958.
[13] S. G. Waley, 'Mechanisms of Organic and Enzymic Reactions', Oxford Univ. Press, 1962.

equation refers to the hydrolysis of a peptide link in nature, much mechanistic

$$RCOX + HY \rightleftharpoons HX + RCOY \quad (1)$$

data for proteases is derived from studies on model substrates, such as esters and amides where R is not necessarily an amino-acid side-chain. Model substrates are often employed because protein hydrolyses cannot be followed conveniently nor can the results of such a study be analysed readily; even peptide hydrolysis is not easily followed. Care is necessary in extrapolating results obtained by use of model substrates to the mechanism of protein hydrolysis.

Proteases are active catalysts at neutral pH, at room temperature, and in aqueous solutions; the Michaelis–Menten rate law is obeyed ([E] < [S]/10).[14]

$$\text{initial rate} = [E][S]\, k_0/(K_m + [S]) \quad (2)$$

We shall denote *mechanism* (*A*) as involving an acyl-enzyme intermediate; the simplest kinetic scheme incorporating this is shown in equations (3) and (4).

$$EH + RCOX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EH{\cdot}RCOX \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ECOR + HX \quad (3)$$

$$ECOR + H_2O \underset{k_{-3}\ R}{\overset{k_3}{\rightleftharpoons}} RCO_2H + EH \quad (4)$$

Kinetic analysis of this scheme on the basis of steady-state assumptions (and $k_{-2} = k_{-3} = 0$ at $t = 0$) yields the Michaelis–Menten rate law [equation (2)] where

$$k_0 = k_2k_3/(k_2 + k_3),\ K_s = (k_2 + k_{-1})/k_1 \quad (5)$$

$$K_m = (k_3/k_1)\,(k_1 + k_{-1})/(k_2 + k_3) \quad (6)$$

Mechanism (*B*) is kinetically simpler than (*A*), but involves no acyl-enzyme; the enzyme–substrate complex breaks down to yield products in a concerted fashion

$$E + RCOX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}RCOX \overset{HY(k_2)}{\rightleftharpoons} E + RCOY + HX \quad (7)$$

A. Enzyme Normality.—The kinetic differential equations for mechanism (*A*) can be solved[15,16] if [S]/10 > [E]. When t is large, the concentration of the first product, [HX], is related to time according to equation (8);

$$[HX] = Vt + P \quad (8)$$

[14] O. H. Strauss and A. Goldstein, *J. Gen. Physiol.*, 1943, **26**, 559.
[15] M. L. Bender, F. J. Kézdy, and F. C. Wedler, *J. Chem. Educ.*, 1967, **44**, 84.
[16] M. L. Bender, M. L. Bégue-Cantón, R. L. Blakely, L. J. Brubacher, J. Feder, C. R. Gunter, F. J. Kézdy, J. V. Killheffer, jun., T. H. Marshall, C. G. Miller, R. W. Roeske, and J. K. Stoops, *J. Amer. Chem. Soc.*, 1966, **88**, 5890,

$$P = [E]\left[k_2/(k_2 + k_3)\right]^2/(1 + K_m/[S])^2 \quad (9)$$

V is identical with the steady-state rate [equation (2)] and the intercept (P) on the time ordinate is diagnostic for mechanism (*A*). Since the intercept is proportional to enzyme concentration this type of experiment can be used to determine enzyme normality.

Table 1 *Some enzyme titrations*

Enzyme	*Substrate*
Chymotrypsin	Acetyl, *N*-Z-L-tyrosyl, isobutyryl, *N*-acetyl-DL-tryptophyl, trimethylacetyl, cinnamoylimidazole, phenylmethanesulphonyl fluoride, *p*-trimethylammoniocinnamoyl,[a] 2,2-dimethyl-5-phenyloxazolinone[b]
Trypsin	Acetyl, 2,4-dinitrophenyl acetate, *N*-acetyl-L-leucyl, *N*(2)-Z-L-lysyl, *p*-guanidinobenzoyl,[c] *N*(2)-acetyl-*N*(1)-benzylcarbazoyl,[d] *N*(2)-methyl-*N*(2)-tosyl-L-lysine naphthyl ester,[d] *N*-acetyl-DL-tryptophyl
Papain	*N*-Z-L-Tyrosyl, *N*-acetyl-DL-tryptophyl, 2,2-dimethyl-5-phenyloxazolinone[b]
Thrombin	*N*(2)-Z-L-Lysyl[e]
Cholinesterase	*o*-Nitrophenyl dimethylcarbamate.
Elastase	Diethyl nitrophenyl phosphate.
Bacterial protease	Acetyl, cinnamoylimidazole.
Thiolbacterial protease	Acetyl[f]

Except where stated, Table shows acyl group of *p*-nitrophenyl ester; Z = benzyloxycarbonyl; [a]J. R. Knowles and J. M. Preston, *Biochem. Biophys. Acta.*, 1968, **151**, 290; [b]J. de Jersey, M. T. C. Runnegar, and B. Zerner, *Biochem. Biophys. Res. Comm.*, 1966, **25**, 383; [c]T. Chase, jun., and E. Shaw, *Biochem. Biophys. Res. Comm.*, 1967, **29**, 508; [d]D. T. Elmore and J. J. Smyth, *Biochem. J.*, 1968, **107**, 97; 103; [e]F. J. Kézdy, L. Lorand, and K. D. Miller, *Biochemistry*, 1965, **4**, 2302; [f]L. Polgar and M. L. Bender, *Biochemistry*, 1967, **6**, 610; other titrations are taken from ref. 16.

The titration must be related observationally with the active site by inhibition experiments for a rigorous demonstration of mechanism (*A*); the intercept is also related to substrate concentration according to equations (10) and (11).

$$1/P^{\ddagger} = C/[S] + C/K_m \quad (10)$$

$$C = K_m\ (k_2 + k_3)/[E]^{\ddagger}k_2 \quad (11)$$

Other experiments (described in more detail later) must be performed to eliminate the *co-existence* of mechanism (*B*) because this does not affect the *qualitative* observation of a titration. Criteria for titrations and for observable intercepts are that $[E] < [S]/10$,* $[S] > K_m$, $k_2 > k_3$, and [E] is greater than the experimental

* This represents the smallest value of [S]/[E] for which equations (8), (9), (10), and (11) are valid.

error in measuring HX. Evidence for mechanism (*B*) is *not* provided by the absence of a titration; in these circumstances indirect methods must be used to decide the mechanism or to titrate the normality.

B. The Pre-steady-state.—Conventional steady-state kinetic methods are not able to dissect enzymatic reactions into their individual rate constants. When these rates are slow such an analysis is possible but only through observations made during the time prior to the steady state. Since the 'pre-steady state' is often very transitory, only fast-reaction techniques are really useful, and these have only recently been applied[11] owing to the availability of commercial instruments. 'Stopped-flow' and rapid-sampling techniques are extensions of existing conventional methods; chemical relaxation methods[17,18] (involving instantaneous physical perturbation to a system) are very flexible in that they cover relaxation times (related to rate constants) ranging from 10^{-10} to 10^{0} sec. An important application of chemical relaxation is the kinetic observation of conformational changes, which are usually rapid processes. A recent example is the interaction between proflavine and α-chymotrypsin;[19] the system shows a chemical relaxation spectrum characteristic of two reactions—the physical binding of the proflavine to the active site and a subsequent conformational change.

C. Catalytic Activity.—There is a limited number of ways in which reactions of the carboxylic acid derivatives can be catalysed.[20] Accordingly, the number of modes of enzymatic catalysis is small. The rate of nucleophilic substitution at the carbonyl of a carboxylic acid derivative is affected[20] by electron-withdrawing groups on the acyl group and the ether oxygen (or amide nitrogen); the rate is increased by a Lewis acid withdrawing electrons from the ether oxygen (or amide nitrogen) or the carbonyl oxygen. The action of a base on the reacting nucleophile (*e.g.* water) will also increase the rate of reaction. Freezing the substrate in a conformation which resembles the transition-state for reaction enhances the reaction rate and this can be accomplished by areas on the enzyme-attracting groups on the substrate. Stereospecificity can be explained if the attraction for one substrate aligns the molecule unfavourably for reaction whilst the other isomer is aligned in a favourable conformation. In general terms, if a series of compounds are substrates, then the better the binding the faster the catalysis.[21]

2 Trypsin

This enzyme (*M*, 23,800) is closely similar to α-chymotrypsin in both structure and mechanism.[1,2] It is (in contrast with α-chymotrypsin) active towards positively charged substrates and is used in peptide sequence analysis because it

[17] M. Eigen and G. G. Hammes, *Adv. Enzymol.*, 1963, **25**, 1.
[18] M. Eigen and L. De Maeyer, 'Investigation of Rates and Mechanisms of Reactions', ed. S. L. Friess, E. S. Lewis, and A. Weissberger, Interscience, New York, 1963.
[19] B. H. Havsteen, *J. Biol. Chem.*, 1967, **242**, 769.
[20] M. L. Bender, *Chem. Rev.*, 1960, **60**, 53.
[21] J. R. Knowles, *J. Theor. Biol.*, 1965, **9**, 213.

will hydrolyse a protein at the lysyl and arginyl residues to give a limited number of peptides.

A. Acyl-enzyme Intermediate and Active Site.—Titration studies provide good evidence for mechanism (*A*) (see Table 1) and the alternative pathway is unlikely because the same normality can be obtained for a particular trypsin solution by use of a variety of different titrators.[16]

The acyl-enzyme intermediate in the trypsin-catalysed hydrolysis of cinnamoylimidazole can only be prepared *in situ*[22] owing to the low k_2/k_3 ratio. Trypsin is added in large excess to cinnamoylimidazole in buffer solution and the ultraviolet absorbence is measured (with use of a blank to correct for enzyme absorption). The disappearance of substrate (followed at 335 mμ) obeys a first-order rate law and is a measure of acylation; the appearance of cinnamate ion (250 mμ) measures deacylation. Deacylation is a relatively slow process and a rapid scan of the spectrum after acylation has occurred yields the ultraviolet spectrum of the intermediate. Comparison with the spectra of model cinnamoyl derivatives suggests the site of acylation to be oxygen or imidazole. Moreover, the denatured intermediate (and that from α-chymotrypsin) hydrolyse at a rate similar to that for model oxygen esters.

Inhibition by phosphorofluoridates gives rise to phosphoryl-trypsins[23] which do not hydrolyse readily; by analogy with this process substrates could hydrolyse *via* an acyl-enzyme intermediate. In contrast with phosphoryl-trypsin the

Table 2 *Ultraviolet absorption maxima for some acyl-enzymes*

Acyl group	*Imidazole*	*Chymotrypsin*	*Methanol*	*Bacterial protease*
Cinnamoyl	307	292 (280)	280	289 (280)
Furoyl	284	256	255	262
Benzoyl	243	272	272	—
Furylacryloyl	340	320 (309)	306	323 (309)
Indoleacryloyl	378	359 (334)	329	358 (334)

Cinnamoyl-trypsin[a]	296
-ficin[b]	330
-papain[c]	326 (305)
-thiosubtilisin[d]	310
-cysteine[c]	306

Wavelengths in mμ; numbers in parenthesis represent λ_{max} in 'denaturing solvents'; data from M. L. Bender and F. J. Kézdy, *Ann. Rev. Biochem.*, 1965, **34**, 49; S. A. Bernhard, S. J. Lau, and H. F. Noller, *Biochemistry*, 1965, **4**, 1108; [a]M. L. Bender and E. T. Kaiser, *J. Amer. Chem. Soc.*, 1962, **84**, 2556; [b]A. Williams, D.Phil. Thesis, Oxford Univ., 1964; [c] M. L. Bender and L. J. Brubacher, *J. Amer. Chem. Soc.*, 1964, **86**, 5333; [d]L. Polgar and M. L. Bender, *Biochemistry*, 1967, **6**, 610.

[22] M. L. Bender and E. T. Kaiser, *J. Amer. Chem. Soc.*, 1962, **84**, 2556.
[23] B. S. Hartley, *Ann. Rev. Biochem.*, 1960, **29**, 45.

acyl-enzyme hydrolyses readily and hence its presence cannot easily be demonstrated.

The identity of k_0 for a series of derivatives of a common acid is good evidence for an intermediate [mechanism (*A*)] and that $k_2 > k_3$ [equation (5)]. This evidence is valid only if the series of leaving groups covers a wide range of reactivity;[24] a constant k_0 could reflect a rate-limiting conformational change in the enzyme. The value of k_0 is constant for derivatives of *N*-benzoyl-L-arginine (methyl, ethyl, isopropyl, cyclohexyl, and benzyl esters[25]) and for a series of benzyloxycarbonyl-L-lysine esters (*p*-nitrophenyl, methyl, and benzyl[26]). A titration has been observed in the hydrolysis of *N*-benzoyl-L-arginine ethyl ester by use of a rapid-sampling technique.[27] Only 50% of the full titration is observed however and this seems incompatible with the above results; if k_2/k_3 is low k_0 could still appear constant, because P contains a term $[k_2/(k_2 + k_3)]^2$, whereas k_0 depends on $k_2/(k_2 + k_3)$ [see equations (5) and (9)].

Phosphoryl-trypsin can be degraded enzymatically and the phosphorus can be located[28] on serine-183, which is probably the site of acylation; the ultraviolet spectrum of the cinnamoylated enzyme is not opposed to this view.

Histidine is almost certainly close to the active site; a chloro-ketone reagent derived from L-lysine[29] (7-amino-1-chloro-3-tosylamidoheptan-2-one) alkylates the enzyme at the active site and degradation studies suggest that a histidine has been modified. Alkylation depends on a base of pK_a *ca.* 7. 2-Iodoacetamide also alkylates trypsin with an apparent pK_a for inhibition of 6.66 and the rate is decreased in the presence of n-butylguanidine but accelerated in the presence of methylguanidine.[30] 3-*N*-Carboxymethylhistidine can be isolated from the digest of the inhibited enzyme. Methylguanidine prevents the inhibition of the enzyme by phosphorylating reagents (which react with the serine) and an explanation of these results is that methylguanidine covers (or protects) only the reactive serine-183 at the active site, whereas n-butylguanidine covers both histidine and serine-183. The geometry of this arrangement allows the distance between the histidine and the serine to be calculated roughly by use of Dreiding models as 5–8 Å. The specificity of trypsin for cationic substrates is clearly connected with the attraction of the active site for guanidine derivatives.

Trypsin has a primary sequence[31] almost identical with that for chymotrypsin, and it is reasonable that the tertiary structures could be similar. The arrangement of the amino-acids at the active-site in chymotrypsin[5] shows histidine-57 about 5 Å from serine-195. Histidine-46 of trypsin lies in a sequence similar to that of histidine-57 of chymotrypsin and is therefore probably situated in a similar conformation.

[24] G. Lowe and A. Williams, *Biochem. J.*, 1965, **96**, 199.
[25] G. W. Schwert and M. A. Eisenberg, *J. Biol. Chem.*, 1949, **179**, 665.
[26] M. L. Bender and F. J. Kézdy, *J. Amer. Chem. Soc.*, 1965, **87**, 4954.
[27] T. E. Barman and H. Gutfreund, *Biochem. J.*, 1966, **101**, 411.
[28] F. Sanger, *Proc. Chem. Soc.*, 1963, 76.
[29] M. Mares-Guia and E. Shaw, *Fed. Proc.*, 1963, **22**, 528.
[30] T. Inagami, *J. Biol. Chem.*, 1965, **240**, PC3453.
[31] A. P. Ryle, *Ann. Reports*, 1966, **63**, 614.

Table 3 *Some peptide sequences*

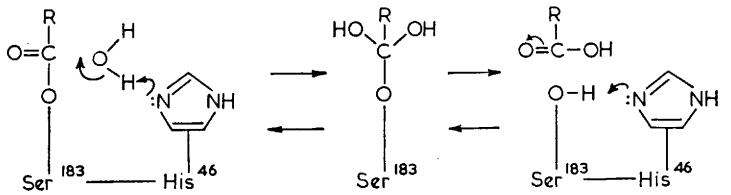
Enzyme	*Peptide sequences* *
Papain[a]	Gly-Ser-C$\overset{*}{y}$s[25]-Trp Val-Asp-His[106] Ile-His[175]-Tyr-Arg
Ficin[b]	Gly-Ser-C$\overset{*}{y}$s
Trypsin[c,d]	Gly-Asp-S$\overset{*}{e}$r[183]-Gly Ala-His[46]-Cys
Thrombin[c,d]	Gly-Asp-S$\overset{*}{e}$r-Gly
Chymotrypsin[c,d]	Gly-Asp-S$\overset{*}{e}$r[195]-Gly Ala-His[57]-Cys
Elastase[c,d]	Gly-Asp-S r-Gly Ala-His-Cys
Streptococcal proteinase[e]	Ala-Ala-Thr-Gly-His-C$\overset{*}{y}$s-Val-Ala
Bacterial proteases 'Novo'[d]	Thr-S$\overset{*}{e}$r-Met-Ala
'Carlsberg'[f]	Leu-Asp(NH_2)-Gly-Thr-S$\overset{*}{e}$r-Met
'Nagarse'[f]	Trp-Asp(NH_2)-Gly-Thr-S$\overset{*}{e}$r-Met
Pepsin[g]	(A$\overset{*}{s}$p, Thr_2, Ser, Gly, Val, Ile, Phe)
Carboxypeptidase A[h,i,j]	Ser-Pro-Cys-Ser Ile-T$\overset{*}{y}$r-Glu(NH_2)-Ala
Carboxypeptidase B[k]	Pro-Thr-Cys-Glu

* There is good evidence that this residue is within the locus of the active site; the cysteine peptides from the carboxypeptidases are not at the active site (see text); sequence is uncertain when residues are in parenthesis; histidine has also been implicated [a]at the active sites of ficin and bromelain; [a]G. Lowe, *Nature*, 1966, **212**, 1263; A. Light, R. Frater, J. R. Kimmel, and E. L. Smith, *Proc. Nat. Acad. Sci. U.S.A.*, 1964, **52**, 1276; S. S. Husain and G. Lowe, *Chem. Comm.*, 1968, 310; [b]R. C. Wong and I. E. Liener, *Biochem. Biophys. Res. Comm.*, 1964, **17**, 470; [c]M. L. Bender and F. J. Kézdy, *Ann. Rev. Biochem.*, 1965, **34**, 49; [d]F. Sanger, *Proc. Chem. Soc.*, 1963, 76; [e]Teh-Yung Liu, W. H. Stein, S. Moore, and S. D. Elliott, *J. Biol. Chem.*, 1965, **240**, 1143; [f]E. L. Smith, F. S. Markland, C. B. Kasper, R. J. DeLange, M. Landon, and W. H. Evans, *J. Biol. Chem.*, 1966, **241**, 5974; [g]R. S. Bayliss and J. R. Knowles, *Chem. Comm.*, 1968, 196; [h]G. N. Reeke, J. A. Hartsuck, M. L. Ludwig, F. A. Quiocho, T. A. Steitz, and W. N. Lipscomb, *Proc. Nat. Acad. Sci. U.S.A.*, 1967, **58**, 2220; [i]K. S. V. Sampath Kumar, K. A. Walsh, J. P. Bargetzi, and H. Neurath, 'Aspects of Protein Structure Symposium', Madras, 1963, p. 319; [j]O. A. Roholt and D. Pressman, *Proc. Nat. Acad. Sci. U.S.A.*, 1967, **58**, 280; [k]E. Wintersberger, *Biochemistry*, 1965, **4**, 1533.

B. Deacylation.—The deacylation of cinnamoyl-trypsin depends on a basic group with pK_a *ca.* 7; acetyl-trypsin shows a similar pH-dependence.[32] Hydrolysis of acyl-chymotrypsins also depends on a basic group of pK_a *ca.* 7 and a comparison shows that the rate constants at limiting pH are identical [k_3 (trypsin)/k_3(chymotrypsin) $= 1 \pm 0{\cdot}5$] over a wide range (10^5) of reactivity.[33] This comparison suggests that deacylation is identical for both enzymes (except insofar as cationic substrates are not compared). The partitioning of the cinnamoyl-enzyme between water and methanol and the deuterium solvent isotope effect on k_3 are very similar; this further supports the identity. The deuterium oxide solvent isotope effect on k_3 is about 2·5 and indicates that the base involved in deacylation (imidazole is the only base available with pK_a *ca.* 7) is acting as a general base rather than as a nucleophile (see section on papain). A minimal mechanism, similar to that for chymotrypsin,[1,2,34] incorporating these ideas is illustrated in

[32] J. A. Stewart and L. Ouellet, *Canad. J. Chem.*, 1959, **37**, 751.
[33] M. L. Bender, J. V. Killheffer, jun., and F. J. Kézdy, *J. Amer. Chem. Soc.*, **1964**, **86**, 5330.
[34] M. L. Bender and F. J. Kézdy, *J. Amer. Chem. Soc.*, 1964, **86**, 3704.

Scheme 1. Acylation, formally the microscopic reverse of deacylation,[34,35] probably follows the reverse of the above pathway except that a nucleophile (HX) will replace water. Interestingly, acylation must involve an extra step; the hydrolysis of *N*-benzoyl-L-arginine *p*-nitroanilide can be followed by a stopped-flow technique,[36] and the results indicate the existence of two non-covalently bound enzyme–substrate complexes (prior to the acyl-enzyme). The first formed enzyme–substrate complex probably undergoes a conformational change prior to acylation of the enzyme.[86]



Scheme 1 *A mechanism for trypsin*

3 Papain

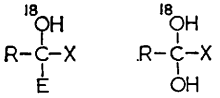
The juice obtained by scratching the surface of papaya fruit contains about 6% of papain.[37] This enzyme (*M*, 21,000) is stored as an inactive mercury derivative and can be regenerated by swirling with a toluene solution of *p*-thiocresol.[38] Structural studies show that the protease consists of a single peptide chain linked by three cystine residues.[39]

A. Acyl-enzyme Intermediate.—The *p*-nitrophenyl esters of *N*-benzyloxycarbonyl-L-tyrosine and *N*-acetyl-DL-tryptophan are able to titrate papain at low pH;[40] the same normality can be obtained by use of either titrant for a given papain solution. 2,2-Dimethyl-5-phenyloxazolinone has also been reported as a titrant for papain.[41]

Esters of hippuric acid show identical values of k_0 (within experimental error) and provide further (but not unequivocal) evidence for a common intermediate,[24] hydrolysis of which is rate determining. Recent work with *N*-benzyloxycarbonylglycine derivatives[42] has confirmed this observation. Methyl and *p*-nitrophenyl hippurates[43] are partitioned identically between water and ethanol during catalysis—again equivocal evidence for mechanism (*A*).

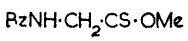
[35] B. Capon and C. W. Rees, *Ann. Reports*, 1963, **60**, 291.
[36] G. Johannin and J. Yon, *Biochem. Biophys. Res. Comm.*, 1966, **25**, 320.
[37] E. L. Smith and J. R. Kimmel, 'The Enzymes', ed. P. D. Boyer, H. A. Lardy, and K. Myrbäck, Academic Press, New York, 1961, vol. 4, p. 133.
[38] M. Soejima and K. Shimura, *J. Biochem.* (*Japan*), 1961, **49**, 260.
[39] A. Light, R. Frater, J. R. Kimmel, and E. L. Smith, *Proc. Nat. Acad. Sci. U.S.A.*, 1964, **52**, 1276.
[40] M. L. Bender and L. J. Brubacher, *J. Amer. Chem. Soc.*, 1966, **88**, 5880.
[41] J. de Jersey, M. T. C. Runnegar, and B. Zerner, *Biochem. Biophys. Res. Comm.*, 1966, **25**, 383.
[42] J. F. Kirsch and M. Igelström, *Biochemistry*, 1966, **5**, 783.
[43] A. C. Henry and F. J. Kirsch, *Biochemistry*, 1965, **4**, 884.

Oxygen-18 studies[44] show that the oxygen of the ester carbonyl of ethyl hippurate is not exchanged with solvent oxygen during catalysis. Mechanism (*A*) predicts this because the intermediate[20,44] (1) previous to the formation of acyl-enzyme is unsymmetrical. However, mechanism (*B*) could give no exchange if the breakdown of (2) is faster in the product direction or if the enzyme surrounds (2) unsymmetrically to prevent the exchange of the oxygen derived from the acyl group.



(1)

(2)



(3)

B. Site of Acylation.—The site of acylation during papain-catalysed reactions could be a thiol group. An observation suggesting this is that the enzyme is inhibited by reagents specific for thiols;[37] the pH-dependence curve for k_0/K_m is bell-shaped for most substrates[37] and the alkaline limb has an apparent pK_a of *ca.* 8 (implying that the thiol reacts as a neutral group rather than an anionic one).

Papain can be cinnamoylated by cinnamoylimidazole and the acyl-enzyme isolated.[45] Comparison of the difference ultraviolet spectrum with the absorption maxima for model compounds suggests either a thiol or an imidazole as the site of acylation. Unequivocal evidence[46,47] has been provided for both the site of acylation and the existence of an acyl-enzyme intermediate; *O*-methyl thiohippurate (3) a substrate of the enzyme, should form a thioacyl-enzyme during hydrolysis. An absorption maximum (313 mμ) appears during hydrolysis and this is characteristic of a dithioester (a slight red shift is observed).

Table 4 *Ultraviolet absorption maxima for O-thioacyl derivatives*

RCS—X[a]	$\lambda_{max.}$ (mμ)
RCS—OR	230 (4·3)
RCS—O^-	249 (2·8)
RCS—NH_2	268 (4·1)
RCS—N·CH:CH·N:CH	250 (4·0)
RCS—SR	305 (4·1)
RCS—papain	313 (4·2)
RCS—ficin	315 (3·7)
RCS—bromelain[b]	315

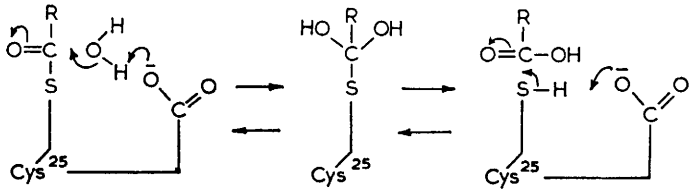
Figures in parentheses refer to $\log_{10} \epsilon_{max}$; [a]G. Lowe and A. Williams, *Biochem. J.*, 1965, **96**, 189; [b]K. Brocklehurst, E. M. Crook, and C. W. Wharton, *Chem. Comm.*, 1967, 1185.

[44] J. F. Kirsch and E. Katchalski, *Biochemistry*, 1965, **4**, 884.
[45] M. L. Bender and L. J. Brubacher, *J. Amer. Chem. Soc.*, 1964, **86**, 5333.
[46] G. Lowe and A. Williams, *Biochem. J.*, 1965, **96**, 189.
[47] G. Lowe and A. Williams, *Proc. Chem. Soc.*, 1964, 140.

The position of the acylated thiol (a cysteine residue) can be determined by use of a specific irreversible inhibitor, 3-chloro-1-tosylamidoacetone (derived from *N*-tosylglycine). The structure of this reactive chloro-compound is similar to that of a substrate, methyl tosylglycinate; the inhibitor therefore has binding characteristics similar to those of the substrate and could bind specifically at the active site. The inhibitor alkylates papain 1,000 times faster than chloroacetone (a chloro-ketone of similar chemical reactivity) and the rate can be decreased by addition of substrates.[48] The inhibitor therefore binds at the active site and subsequently reacts with an adjacent nucleophile. Degradation of the enzyme alkylated with ^{14}C-labelled inhibitor indicates[49] cysteine-25 as the reactive thiol. Further evidence for the existence of a thiol group at the active site is the dependence[40] of the rate of inhibition by the chloro-ketone derived from *N*-tosyl-L-phenylalanine on a basic group of pK_a 8·28.

Recent work, with a 'bridging' reagent, dibromoacetone, indicates that a histidine is located close to cysteine-25 at residue 106 instead of 175 as previously thought;[50] moreover the dimensions of the 'bridging' reagent suggest that the distance between the residues is about 5 Å.* This interesting result should be compared with that for streptococcal proteinase (see later).

C. Deacylation.—The pH-dependence of k_3 (apparent pK_a *ca.* 4)[40,45] provides indirect evidence for the participation of a carboxylate anion. The deuterium oxide solvent isotope effect on deacylation (2·5)[45] is approximately that expected for general base catalysis and a 'minimal' scheme for deacylation is shown (Scheme 2) (the function of the histidine close to the active thiol is not yet known



Scheme 2 *A 'minimal' mechanism for papain*

and this species is therefore not included in the mechanism). The partitioning of the acyl-enzyme between nucleophiles and water does not follow any basicity pattern,[45] which implies that the nucleophile possesses a binding site on the enzyme. For example D-tryptophanamide reacts 200 times more slowly than the L-form with cinnamoyl-papain.[45]

* The recently published *X*-ray structure for papain (J. Drenth, J. N. Jansonius, R. Koekoek, H. N Swen, and B. G. Wolthers, *Nature*, 1968, **218**, 929) is in complete agreement with this result.

[48] A. Williams, D.Phil. Thesis, Oxford Univ., 1964.
[49] S. S. Husain and G. Lowe, *Chem. Comm.*, 1965, 345.
[50] G. Lowe, *Nature*, 1966, **212**, 1263.

D. Acylation.—The ratio k_0/K_m (which is equivalent to k_2/K_s) for the hydrolysis of aryl hippurates obeys a Hammett relationship[24] with a sensitivity + 1·27 towards the σ constants obtained from the ionisation of benzoic acids. Acylation therefore involves a nucleophile, but there can be no appreciable ionisation of the phenol in the transition state. This and the relatively small sensitivity support a mechanism where an acid acts as a catalyst. The separation of binding (K_s) from acylation (k_2) is not possible (except by the use of fast-reaction techniques) but the change in k_0/K_m probably reflects a change in k_2, since there is no appreciable structural change in the substrate. A more general linear free energy relationship exists between k_0/K_m for any hippuric acid derivative and the rate constant for the reaction of hydroxide ion with the corresponding acetyl derivative.[51] It is possible to dissect the Michaelis–Menten parameters for the substrate isopropyl hippurate on the assumption that the mechanism can be represented by equations (3) and (4), and that k_0 for the methyl ester is equal to k_3.[24] The bell shaped pH-dependence for k_0/K_m for this substrate reflects that of K_s; k_2 is pH-independent from pH 4·5 to 8·5.[52] The acylation mechanism is probably the microscopic reverse of deacylation where a nucleophile (HX) replaces water.

4 Ficin

This enzyme (M, 26,000) occurs in many species of fig; it consists of a single protein chain but only portions of its sequence are known. Its properties resemble those of papain and a thiol group is implicated in its mechanism.[37]

O-Methyl thiohippurate, a substrate for ficin, yields a transient ultraviolet absorption maximum at 315 mμ when mixed with the enzyme;[46,47] cinnamoylimidazole also acylates ficin and the spectrum of the intermediate is similar to that for the papain derivative.[48] *p*-Nitrophenyl hippurate can titrate the enzyme[53] and esters of *N*-benzoyl-L-arginine have identical values for k_0.[54] These observations are all in accord with mechanism (*A*), although no experiment has yet excluded the co-existence of mechanism (*B*).

The site of acylation is clearly a thiol and this can be identified by use of [^{14}C]-iodoacetamide.[55] The sequence around the active thiol of ficin is closely similar to that in papain, and the kinetics of alkylation by iodoacetamide and chloroacetamide[56] depend on a basic group of pK_a 8·6.

Direct studies of deacylation have not been made but values of k_0 for *N*-benzoyl-L-arginine derivatives (k_3) depend on a basic group with pK_a *ca.* 3·5,[54] suggesting the involvement of a carboxylate anion. The kinetics of acylation are also not directly available, but k_0/K_m depends on a basic and an acidic group of pK_a *ca.* 4 and 8 respectively. Comparison with papain indicates that this pH-dependence probably reflects that of binding. The apparent pK_a values for

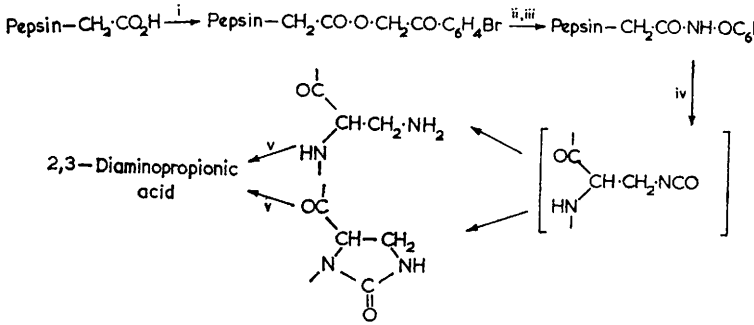
[51] A. Williams, quoted in ref. 1.
[52] Unpublished work with E. C. Lucas.
[53] M. R. Hollaway and H. Gutfreund, quoted in A. W. Lake and G. Lowe, *Biochem. J.*, 1966, **101**, 402.
[54] B. R. Hammond and H. Gutfreund, *Biochem. J.*, 1959, **72**, 349.
[55] R. C. Wong and I. E. Liener, *Biochem. Biophys. Res. Comm.*, 1964, **17**, 470.
[56] M. R. Hollaway, A. P. Mathias and B. R. Rabin, *Biochim. Biophys. Acta.*, 1964, **92**, 111.

k_0/K_m are the same for most substrates.[37] The data at present available for ficin suggest a mechanism identical with that for papain.

5 Pepsin

Pepsin[31] (*M*, 34,000) is found in the gastric juices of mammals; it is an acidic protein active at a low pH (2–3) and has a high selectivity towards peptides of L-amino-acids although the hydrolysis of esters is weakly catalysed.

A. The Active Site.—A carboxy-group almost certainly resides within the locus of the active site and takes part in the catalytic mechanism. Pepsin can be inactivated (1:1 stoicheiometry) in the presence of cupric ions by the diazo-ketone derived from L-phenylalanine (1-diazo-3-tosylamido-4-phenylbutan-2-one).[57] This reaction is characteristic of a carboxy-group[58] and nucleophiles such as potassium iodide can reactivate the inhibited material, which suggests that an ester has been formed. The inhibitor derived from D-phenylalanine reacts only slowly and both inhibitions are prevented by the presence of substrate. The altered carboxy-residue is probably not the C-terminal alanine, because the enzyme inhibited with methyl *N*-diazoacetyl-L-leucinate loses its *C*-terminal on treatment with carboxy-peptidase.[59] Another set of observations[60] also indicates the existence of a carboxy-group at the active site; the isolation of 2,3-diaminopropionic acid from the amino-acid digest[60] suggests that an aspartic acid residue has been alkylated. A peptide can be isolated[61] from pepsin inhibited by methyl *N*-diazoacetyl-L-phenylalaninate (1:1 stoicheiometry); the radioactive inhibitor is probably attached to an aspartyl residue.



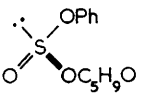
Scheme 3 *Reagents: i,* p-*bromophenacyl bromide; ii, hydroxylamine; iii,* 2,4-*dinitrofluorobenzene; iv, Lossen rearrangement; v,* H_3O^+, 110°, 24 hr.

Kinetic studies with pepsin have been hampered by the absence of a convenient technique for following peptide hydrolyses; some peptides (*e.g. N*-acetyl-L-

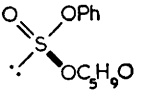
[57] G. R. Delpierre and J. S. Fruton, *Proc. Nat. Acad. Sci. U.S.A.*, 1966, **56**, 1817.
[58] H. Zollinger, 'Diazo and Azo Chemistry', Interscience, New York, 1961.
[59] T. G. Rajagopalan, S. Moore, and W. H. Stein, *J. Biol. Chem.*, 1966, **241**, 4940.
[60] E. Gross and J. L. Morell, *J. Biol. Chem.*, 1966, **241**, 3638.
[61] R. S. Bayliss and J. R. Knowles, *Chem. Comm.*, 1968, 196.

phenylalanyl-L-tyrosine) undergo a change in their ultraviolet absorption spectra on hydrolysis.[62] Although the kinetics of these reactions have been carefully investigated, no definite proposals for a mechansim are available. A potentially important discovery is that pepsin catalyses the hydrolysis of organic sulphite esters.[63] The reaction is a genuine catalysis associated with Michaelis–Menten parameters (k_0 and K_m) and the active site for peptide hydrolysis; k_0 for the hydrolysis of methyl phenyl sulphite depends on an acidic group with an apparent pK_a of 2·6. However, peptide substrates show a bell-shaped profile[62] with a maximum at pH *ca.* 3. The very low deuterium oxide solvent isotope effect on k_0 for both sulphite and peptide substrates suggests that a proton is not involved in either a pre-equilibrium or a rate-controlling step. It has been suggested[1,64] that a carboxylate ion acts as a nucleophile; this implies the existence of an anhydride intermediate.

It is interesting that the catalysis of sulphite ester hydrolysis is stereospecific;[63] pepsin has been used to resolve asymmetric sulphites and the resolution of phenyl furfuryl sulphite [formulae (4) and (5)] is a specific example of this.[63]



(4)



(5)

6 Bacterial Proteases

Bacteria and moulds can excrete proteolytic enzymes into their growth media. These enzymes generally have a wide specificity towards amides and esters and can even catalyse the hydrolysis of alkyl esters of fatty acids.[65] Use is made of this wide specificity in the dry cleaning industry where bacterial proteases are used to remove protein stains.

The family of proteases (M, 28,000) from strains of *Bacillus subtilis* are structurally very similar;[66] they consist of a single protein chain and contain no cysteine residues. Substrates such as *N*-cinnamoylimidazole and *p*-nitrophenyl acetate can titrate the enzymes during the attainment of the steady state.[16,67] Cinnamoyl-proteases can be isolated[68] (although they deacylate fairly readily) and phosphorofluoridates inhibit the enzyme. The enzyme can also be titrated with toluene-*p*-sulphonyl fluoride[67] and, like dephosphorylation, desulphonylation is slow.

[62] J. L. Denburg, R. Nelson, and M. S. Silver, *J. Amer. Chem. Soc.*, 1968, **90**, 479.
[63] T. W. Reid and D. Fahrney, *J. Amer. Chem. Soc.*, 1967, **89**, 3941; T. W. Reid, T. P. Stein, and D. Fahrney, *ibid.*, p. 7125.
[64] G. E. Clement and S. L. Snyder, *J. Amer. Chem. Soc.*, 1966, **88**, 5338.
[65] B. Hagihara in ref. 37, p. 193.
[66] E. L. Smith, F. S. Markland, C. B. Kasper, R. J. DeLange, M. Landon, and W. H. Evans, *J. Biol. Chem.*, 1966, **241**, 5974.
[67] L. Polgar and M. L. Bender, *Biochemistry*, 1967, **6**, 610.
[68] S. A, Bernhard, S. J. Lau, and H. F. Noller, *Biochemistry*, 1965, **4**, 1108; H. F. Noller and S. A. Bernhard, *ibid.*, 1118.

Degradation of the phosphoryl[69] and the furylacryloyl[68] enzymes indicated that a serine had been altered. Cinnamoyl- and furylacryloyl-proteases, isolated at low pH[68] have ultraviolet spectra characteristic of either an oxygen ester or an imidazole amide. Denaturation with sodium dodecyl sulphate yields spectra characteristic of oxygen esters. Serine is therefore a likely candidate for the site of acylation during hydrolysis (see Table 2).

Kinetics for bacterial proteases are similar to those for α-chymotrypsin and trypsin; k_0 follows the ionisation of a base with pK_a *ca.* 7, but K_m is pH-independent; high values of K_m are in accord with the low specificity of the enzyme. In contrast with the mammalian proteases such as trypsin these enzymes are not inhibited by chloro-ketones related to amino-acids.[66]

$$\text{E—CH}_2\text{OH} \xrightarrow{\text{i}} \text{E—CH}_2\cdot\text{O}\cdot\text{SO}_2\cdot\text{CH}_2\cdot\text{Ph} \xrightarrow{\text{ii}} \text{E—CH}_2\cdot\text{S}\cdot\text{CO}\cdot\text{Me} \xrightarrow{\text{iii}} \text{E—CH}_2\cdot\text{SH}$$

Scheme 4 *Reagents:* i, *phenylmethanesulphonyl fluoride;* ii, *sodium thioacetate;* iii, *neutral* p*H*

The protease from a Danish strain can be sulphonylated at serine-220,[67] and treatment with thioacetate ion substitutes an acetyl thiol for the original hydroxy-group. At about pH 7 the modified enzyme loses the acetyl group to give a thiol-enzyme. The modified protein is still active as a catalyst[67] but K_m and k_0 values for the substrate p-nitrophenyl acetate are much lower than in the oxygen analogue. The enzyme is now inhibited by reagents acting specifically for thiols but sulphonyl fluorides are no longer effective inhibitors. The enzyme can also be cinnamoylated with cinnamoylimidazole and the intermediate has an ultraviolet absorption maximum at about 310 mμ, in agreement with the formation of a thiol-ester. The pH-dependence for K_m is also changed although that for k_0 remains roughly the same for *p*-nitrophenyl acetate.

7 Streptococcal Proteinase

Some streptococci excrete an inactive protein[70] into their culture media; the protein (*M*, 44,000) can be converted into an active protease (*M*, 32,000) on treatment with a reducing agent and trypsin. The enzyme shows a wide specificity towards carboxylic acid derivatives and contains only one cysteine residue,[71] which is essential for catalysis.

The protease can be alkylated (and inhibited) by chloroacetamide, and the alkylation rate depends on a basic group[72] with pK_a *ca.* 9; alkylation by the corresponding acid is much faster and depends on both a base (pK_a *ca.* 5) and an acid (pK_a *ca.* 9).

Papain shows a similar difference in the pH-dependences for alkylation by 2-halogeno-acids and amides.[73] There are few similarities between the synthetic

[69] R. A. Oosterbaan, P. Kunst, J. Van Rotterdam, and J. A. Cohen, *Biochim. Biophys. Acta.*, 1958, **27**, 556; R. A. Oosterbaan and M. E. Van Andrichem, *ibid.*, p. 423.
[70] Teh-Yung Liu and S. D. Elliott, *J. Biol. Chem.*, 1965, **240**, 1138, and references quoted therein.
[71] Teh-Yung Liu, W. H. Stein, S. Moore, and S. D. Elliott, *J. Biol. Chem.*, 1965, **240**, 1143.
[72] B. I. Gerwin, *J. Biol. Chem.*, 1967, **242**, 451.
[73] K. Wallenfels and B. Eisele, *European J. Biochem.*, 1968, **3**, 267.

thiol-subtilisin[67] and streptococcal proteinase (only one cysteinyl residue, no disulphide links and similar kinetics[74]).

The thiol can be blocked to reaction with electrophiles by sodium tetrathionate, although the blocked enzyme still binds substrate. The blocked enzyme reacts with 2-*N*-bromoacetyl-L-arginine methyl ester, a substrate analogue, and removal of the tetrathionate with reducing agents reveals inactive protein; degradation of the altered enzyme shows that a histidine has been modified.[75]

The parameter k_0 depends on a basic group of pK_a 6·4;[74] this group could be the histidine previously mentioned, and if this is true its function is more apparent than is that of the histidine in papain, and it could act in the mechanism of α-chymotrypsin or trypsin catalyses. The function of the thiol is less certain and no definitive evidence has yet been published for the participation of a thiol acyl intermediate.

8 Metal-ion-potentiated Proteases

Reactions of nucleophiles with peptides are well known to be catalysed by transition-metal ions.[1,76] The driving force of these catalyses is removal of electrons from the carboxy-group in a metal ion complex. Whereas the driving force in metal-free proteases is not thoroughly understood (general base catalysis and 'freezing' the substrate in a particular conformation by hydrophobic and hydrogen bonding are the only well documented pathways) that for carboxypeptidase, a 'zinc' enzyme is definitely the formation of a co-ordination complex.

Carboxypeptidase A.—Carboxypeptidase A (*M*, 34,300) consists of a single peptide chain; it contains one zinc atom per molecule,[77] which can be removed by dialysis against a solution of 1,10-phenanthroline. The metal-free protein (*apo*-enzyme) is no longer active, but the crystalline structure is unchanged.[78] Addition of substrate to the *apo*-enzyme prevents reactivation by zinc ion.[79] The *apo*-enzymes from carboxypeptidases A and B (enzymes of different specificity) can react with reagents specific for thiol (*N*-ethylmaleimide), and the altered protein cannot be reactivated by zinc.[80,81] These reagents, however, do not react in the presence of a typical substrate, a reversible inhibitor, or zinc ions; degradation of the modified protein yields a peptide containing altered cysteine. It was suggested on the basis of the above evidence, that the thiol was close to the active sites of both enzymes and that the sulphur was co-ordinated to the zinc ion.[82]

[74] B. I. Gerwin, W. H. Stein, and S. Moore, *J. Biol. Chem.*, 1966, **241**, 3331.
[75] Teh-Yung Liu, *J. Biol. Chem.*, 1967, **242**, 4029.
[76] M. L. Bender, A.C.S. Advances in Chemistry Series, 1963, vol. **37**, p. 19; M. L. Bender and R. Breslow, 'Comprehensive Biochemistry', ed. M. Florkin and E. H. Stotz, Elsevier, New York, 1962.
[77] B. L. Vallee and H. Neurath, *J. Amer. Chem. Soc.*, 1954, **76**, 5006.
[78] H. Neurath, in ref. 37, p. 11.
[79] B. L. Vallee, J. A. Rupley, T. L. Coombs, and H. Neurath, *J. Biol. Chem.*, 1960, **235**, 64.
[80] K. S. V. Sampath Kumar, K. A. Walsh, J. P. Bargetzi, and H. Neurath, Aspects of Protein Structure Symposium, Madras, 1963, p. 319.
[81] E. Wintersberger, *Biochemistry*, 1965, **4**, 1533.
[82] B. L. Vallee, T. L. Coombs, and F. L. Hoc, *J. Biol. Chem.*, 1960, **235**, Pc 45.

Chemical studies have also shown that a tyrosine residue is close to the active site.[83]

9 Tertiary Structures of Proteases

The tertiary structures of a tosyl derivative of α-chymotrypsin and carboxypeptidase A at about 2 Å resolution are already available.[5,6] The structure of papain is known at 6 Å resolution and a more refined model is close to publication.[84]

Structural studies can never solve completely the mechanistic problems of enzyme catalysis. Indeed the mechanisms of many reactions of organic compounds of known structure are not yet fully understood. However, the importance of structural data to the elucidation of mechanism can never be overestimated; it is possible, by use of *X*-ray data, to eliminate certain mechanistic hypotheses on the basis of structure alone. For example, both histidines (40 and 57) have been implicated in a concerted mechanism for α-chymotrypsin catalysis;[34] however, the model derived from *X*-ray data[5] shows that histidine-40 is far enough away (*ca.* 13 Å) from histidine-57 for this to be impossible. There is no observable cleft in the protein structure where a substrate can bind (in contrast to lysozyme[3] and ribonuclease[4,7]). A salt bridge exists between aspartate-194 and the *N*-terminal isoleucine-16;[5] this link resides in a hydrophobic region and the ammonium ion is deprotonated as the pH is raised, to leave an anion in an unfavourable environment. A change in conformation could relieve this strain and at the same time prevent the binding of substrate; the observed kinetics show that K_S does rise around pH 9, corresponding to the ionisation of the isoleucine.[85] The overall similarity in primary sequence of α-, π-, γ-, and δ-chymotrypsins and chymotrypsinogen is also reflected in their tertiary structures.[86]

The *X*-ray data for carboxypeptidase A[6] at a resolution of 2·0 Å (and the complex with the substrate *N*-glycyl-L-tyrosine at 2·8 Å) confirm that there is one zinc atom per molecule and that there are 307 amino-acid residues. Zinc has been shown to reside at the active site by difference measurements on the enzyme and enzyme–substrate crystals; an increase in electron density (equivalent to substrate) occurs close to the zinc.[6] The data show zinc in the free enzyme bound to three ligands, one of which is an imidazole residue; photo-oxidation studies,[87] however, have not favoured imidazole as a ligand. A cystine residue is located far from the zinc ion and the surrounding amino-acid sequence identifies this cystine with that postulated to be co-ordinated with the zinc.[80] There is evidence supporting an *N*-terminal amino-acid as a zinc ligand[87] but both *N*- and *C*-terminals are too distant from the zinc in the *X*-ray model for this to occur.[6] The lack of specificity for the side chain of the peptide (in hydrolysis) can be explained,

[83] O. A. Roholt and D. Pressman, *Proc. Nat. Acad., Sci. U.S.A.*, 1967, **58**, 280.
[84] J. Drenth, J. N. Jansonius, and B. G. Wolthers, *J. Mol. Biol.*, 1967, **24**, 449.
[85] M. L. Bender, M. J. Gibian, and D. J. Whelan, *Proc. Nat. Acad. Sci. U.S.A.*, 1966, **56**, 833.
[86] J. Kraut, H. T. Wright, M. Kellerman, and S. T. Freer, *Proc. Nat. Acad. Sci. U.S.A.*, 1967, **58**, 304.
[87] T. L. Coombs, Y. Omote, and B. L. Vallee, *Biochemistry*, 1964, **3**, 653.

since a large volume of low electron density is found close to the zinc in the *X*-ray model. Comparison of enzyme and enzyme–substrate complex shows that there is a 2 Å movement of the guanidine of an arginyl residue towards the carboxylate anion of the substrate on binding, owing to the rotation of the arginyl residue about the C(3)—C(4) bond. Although the peptide link of the substrate is not observed it can be arranged in the model (derived from the *X*-ray data), so that the carbonyl co-ordinates with the zinc.[6] Another group found to be close to the peptide link is probably an aspartyl or glutamyl residue. During binding the phenolic hydroxy-group of a tyrosine residue moves through 14 Å [by rotation about the C(2)—C(3) bond] towards the substrate. This tyrosyl is identified with a sequence known to be close to the active site.[83] This result therefore provides a most striking demonstration of Koshland's theory involving a conformational change induced in the enzyme by the binding of a substrate.[88]

10 Concluding Remarks*

The study of enzymes has in the past been concentrated on function and only recently have *X*-ray and chemical methods allowed meaningful structural investigations to be made. Since detailed knowledge of mechanism requires structural information, future studies of mechanism and structure will be closely allied. The study of enzyme mechanisms is in some respects simpler than studies of mechanisms involving small molecules, because the conformation of the enzyme is relatively rigid and so also is that of the substrate when found at the active site.

* Since this article was written (early 1968) the Royal Society has held a symposium important for its discussion of the structural aspects of proteases.

[88] D. E. Koshland, Cold Spring Harbor, Symposia on Quantum Biology, 1963, vol. 28, p. 473.