ACCOUNTS OF CHEMICAL RESEARCH

VOLUME 7 NUMBER 8 AUGUST, 1974

The Active Site of Pepsin

Joseph S. Fruton

Kline Biology Tower, Yale University, New Haven, Connecticut 06520

Received March 4, 1974

The two general properties of enzymes—their remarkable catalytic efficiency and exquisite specificity—offer some of the most challenging problems in modern chemistry. Although the wide variety of reactions effected by the many known enzymes can be rationalized in terms of a limited number of mechanisms (*e.g.*, general acid–base catalysis, nucleophilic or electrophilic catalysis), the manner in which these catalytic mechanisms are expressed is determined by the specific interaction of the substrate with the active-site groups of the enzyme. Several complementary lines of attack are giving significant knowledge about the active sites of individual enzymes and about the way they interact with their substrates. They are, in addition to the determination of the amino acid sequence of the enzyme protein, the study of its three-dimensional structure by X-ray crystallography, the selective chemical modification of enzymes, the spectroscopic study (*e.g.*, by nuclear magnetic resonance) of enzyme–substrate interaction, and the examination of the effect of structural changes in the substrate on its interaction with the enzyme that catalyzes its transformation. We have used the last approach to study the specificity and the mechanism of action of pepsin and related proteinases by devising peptide substrates whose structure has been varied systematically. We have also attached groups that can act as fluorescent probes for changes in the environment of the substrate molecule. The results obtained thus far have led us to formulate several working hypotheses about the mode of enzyme–substrate interaction in the catalytic process effected by these proteinases.

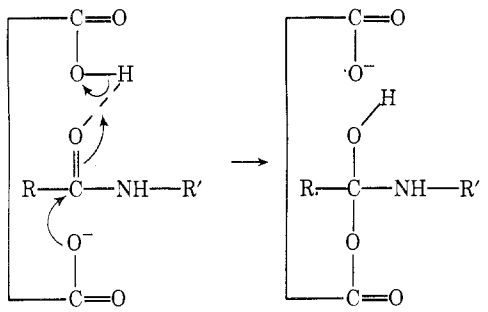
Acid Proteinases

Gastric pepsin A is the best known member of a group of enzymes that cleave proteins in the pH range 2–5, and which are therefore termed "acid proteinases." Other acid proteinases are the milk-clotting enzyme rennin (chymosin), a number of mold enzymes (*e.g.*, penicillopepsin, *Rhizopus* pepsin), and the cathepsin D of the lysosomes of animal tissues. These enzymes appear to have similar catalytic centers, since they are inhibited selectively by active-site-directed reagents such as diazoacetyl-L-phenylalanine ethyl ester and *p*-toluenesulfonyl-L-phenylalanyldiazomethane[1] or diazoacetyl-DL-norleucine methyl ester.[2] In the case of gastric pepsin[3] and penicillopepsin,[4] it has been shown that the site of attack by such diazo compounds is the β-carboxyl group of an aspartyl residue in homologous sequences (pepsin: Ile-Val-Asp-Thr-Gly-Thr-Ser; penicillopepsin: Ile-Ala-Asp-Thr-Gly-Thr-Leu). Moreover, considerable homology has been noted in the amino acid sequences of pepsin and rennin.[5] The complete amino acid sequence of porcine pepsin has been reported recently,[6] and the aspartyl residue sensitive to diazo compounds has been identified as Asp-215. In addition, Asp-32 has been identified as the residue sensitive to epoxides[7] which inhibit pepsin.

These findings are relevant to the problem of the mechanism of pepsin action. Earlier suggestions regarding the mode of pepsin action had invoked the participation of two enzymic carboxyl groups, one acting as a proton donor, the other (in its carboxylate form) as a nucleophile,[8] and various mechanistic proposals have been offered[9] in which a tetrahedral intermediate (I) is involved in the cleavage of a sub-

Joseph S. Fruton is Eugene Higgins Professor of Biochemistry at Yale University. He was born in 1912 in Czestochowa, Poland, and came to the United States in 1923. He received both his B.A. and Ph.D. degrees from Columbia University. From 1934 to 1945, he was on the staff of the Rockefeller Institute for Medical Research, after which he moved to Yale. At Yale, he has served as Chairman of the Department of Biochemistry and as Director of the Division of Science. His research interests are in bioorganic chemistry, with primary emphasis on the specificity and mechanism of enzyme action, peptide chemistry, and transpeptidation reactions catalyzed by proteinases.

(1) G. R. Delpierre and J. S. Fruton, *Proc. Nat. Acad. Sci. U. S.*, 56, 1817 (1966).
(2) T. G. Rajagopalan, W. H. Stein, and S. Moore, *J. Biol. Chem.*, 241, 4295 (1966).
(3) R. S. Bayliss, J. R. Knowles, and G. R. Wybrandt, *Biochem. J.*, 113, 377 (1969).
(4) J. Sodek and T. Hofmann, *Can. J. Biochem.*, 48, 1014 (1970).
(5) V. B. Pederson and B. Foltmann, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, 35, 250 (1973).
(6) J. Tang, P. Sepuvelda, J. Marciniszyn, Jr., K. C. S. Chen, W-Y. Huang, N. Too, D. Liu, and J. P. Lanier, *Proc. Nat. Acad. Sci. U. S.*, 70, 3437 (1973); see also L. Moravek and V. Kostka, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, 35, 276 (1973).
(7) J. A. Hartsuck and J. Tang, *J. Biol. Chem.*, 247, 2575 (1972).
(8) G. R. Delpierre and J. S. Fruton, *Proc. Nat. Acad. Sci. U. S.*, 54, 1161 (1965); T. R. Hollands and J. S. Fruton, *ibid.*, 62, 1116 (1969); J. R. Knowles, *Phil. Trans. Roy Soc. London, Ser. B*, 257, 135 (1970).
(9) M. W. Hunkapiller and J. H. Richards, *Biochemistry*, 11, 2829 (1972); G. E. Clement, *Progr. Bioorg. Chem.*, 2, 177 (1973).



I

strate RCO-NHR′. The further fate of this hypothetical intermediate is a subject of active discussion; we shall return to the problem at the end of this article. We assume, as a working hypothesis, that the acid proteinases related to pepsin operate by similar catalytic mechanisms, thus providing a counterpart to the serine proteinases (chymotrypsin, trypsin, elastase, subtilisin) which involve active-site seryl and histidyl residues.

The Primary Specificity of Pepsin

Until recently, the specificity of proteinases was described in terms of the particular amino acid residues whose participation in a peptide bond rendered that bond most sensitive to the enzyme under study. This view came from the discovery of the first synthetic substrates for well-defined proteinases such as pepsin, trypsin, chymotrypsin, and papain[10] and the subsequent work on the specificity of these enzymes.[11,12] It is now clear, however, that in addition to the "primary" enzyme–substrate interactions involving the amino acid residues forming the sensitive bond of the substrate, there are important "secondary" interactions in which substrate groups relatively distant from the site of catalytic attack combine with the enzyme to produce large effects on its catalytic efficiency.[12] Such secondary enzyme–substrate interactions must obviously be included in a description of the specificity of proteinases and, indeed, of other enzymes that act on oligomeric substrates.

The first synthetic substrates for pepsin[13] such as Z-Glu-Tyr[14] or Ac-Phe-Tyr were rather resistant to enzymic cleavage, but recent work has provided more sensitive substrates, some of which are cleaved at very high rates.[12] For the study of the primary specificity of pepsin, a series of compounds of the type Z-His-X-Phe-OMe and Z-His-Phe-Y-OMe were prepared in which the nature of the amino acid residue at the X or Y position was varied; in all cases the X–Phe or Phe–Y bond was the only one cleaved by the enzyme under the conditions of the study.[15] It was found that with small substrates of the type AX–YB, where the X–Y bond is broken, pepsin exhibits a preference for a L-phenylalanyl residue in the X position and a L-tryptophyl, L-tyrosyl, or L-phenylalanyl residue in the Y position. Replacement of these aromatic amino acids by aliphatic ones (*e.g.*, Leu, Met) bearing hydrophobic side chains or by an alicyclic one (β-cyclohexyl-L-alanine) markedly reduces, but does not abolish, the cleavage of the X–Y bond, except in the case of X = Val or Ile where branching at the β carbon completely inhibits pepsin action. Moreover, pepsin exhibits an absolute requirement for the L enantiomer in both the X and the Y position of substrates such as A-Phe-Phe-B. If either phenylalanyl residue is the D enantiomer, the resulting compound is resistant to pepsin action and acts as a competitive inhibitor with a K_i value approximating the K_m value[16] of the L,L substrate.[17] This indicates that the resistance of the L,D or D,L diastereoisomer is not a consequence of poor binding to the enzyme, but rather one of faulty positioning of the sensitive peptide bond with respect to the catalytic groups of pepsin.

For the determination of the kinetic parameters for the hydrolysis by pepsin of the sensitive CO–NH bond in synthetic peptides, an automatic ninhydrin method[18] was used in earlier work but has been replaced by the recently developed fluorometric method using fluorescamine.[19] Also, advantage was taken of the fact that when the X unit in substrates of the type A-X-Phe-B is the *p*-nitro-L-phenylalanyl residue, the kinetic parameters are similar to analogous substrates of the type A-Phe-Phe-B. The cleavage of the Phe(NO_2)–Phe bond at pH 4 is accompanied by a relatively large change in absorbance at 310 nm, thus providing a useful spectrophotometric method for studying pepsin kinetics.[17] This method has been employed to demonstrate unequivocally the ability of pepsin to act as an esterase, with Z-His-Phe(NO_2)-Pla-OMe as the substrate.[20] It has also been used for the determination of the K_i values for resistant substrate analogs or poor substrates, when these act as competitive inhibitors of the hydrolysis of Z-His-Phe(NO_2)-Phe-OMe.[21] These data indicated that the principal contribution to the binding energy in the interaction of small substrates of the type A-Phe-Phe-B with the active site of pepsin is provided by the Phe-Phe unit. Direct binding studies, by gel filtration,[22] gave strong support to this conclusion.[23]

The substrates mentioned above have a site of protonation at the imidazolyl group, rendering them moderately soluble in aqueous buffered media in the pH range 2–5, and the addition of an organic solvent is not required; such solvents have been shown to inhibit the action of pepsin on synthetic substrates.[24]

(10) M. Bergmann and J. S. Fruton, *Advan. Enzymol.*, 1, 63 (1941).
(11) H. Neurath and G. W. Schwert, *Chem. Rev.*, 46, 69 (1950).
(12) J. S. Fruton, *Advan. Enzymol.*, **33**, 401 (1970).
(13) J. S. Fruton and M. Bergmann, *J. Biol. Chem.*, 127, 627 (1939); L. E. Baker, *ibid.*, **193**, 809 (1951).
(14) Abbreviations used in this article: Z, benzyloxycarbonyl; Phe(NO_2), *p*-nitro-L-phenylalanyl; Pla, β-phenyl-L-lactyl; OP4P, 3-(4-pyridyl)propyl-1-oxy; Dns, dansyl, 5-dimethylamino-1-naphthalenesulfonyl; Mns, mansyl, 6-(*N*-methylanilino)-2-naphthalenesulfonyl; TPDM, *p*-toluenesulfonyl-L-phenylalanyldiazomethane; Aac, 9-aminoacridine. The abbreviated designation of amino acid residues denotes the L form.
(15) G. E. Trout and J. S. Fruton, *Biochemistry*, 8, 4183 (1969).

(16) The kinetic parameters mentioned in this article are defined by the equation $v = V_m(S)/K_m + (S))$ for the process

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2$$

where v = initial velocity, the maximal velocity $V_m = k_{cat} \times$ total enzyme concentration, (S) = initial substrate concentration, $K_m = (k_{cat} + k_{-1})/k_1$, and $K_s = k_{-1}/k_1$.
(17) K. Inouye and J. S. Fruton, *Biochemistry*, 6, 1765 (1967).
(18) T. R. Hollands and J. S. Fruton, *Biochemistry*, 7, 2045 (1968).
(19) P. S. Sampath-Kumar and J. S. Fruton, *Proc. Nat. Acad. Sci. U. S.*, 71, 1070 (1974).
(20) K. Inouye and J. S. Fruton, *J. Amer. Chem. Soc.*, 89, 187 (1967).
(21) K. Inouye and J. S. Fruton, *Biochemistry*, 7, 1611 (1968).
(22) G. F. Fairclough, Jr., and J. S. Fruton, *Biochemistry*, 5, 673 (1966).
(23) R. E. Humphreys and J. S. Fruton, *Proc. Nat. Acad. Sci. U. S.*, 59, 519 (1968); E. V. Raju, R. E. Humphreys, and J. S. Fruton, *Biochemistry*, 11, 3533 (1972).

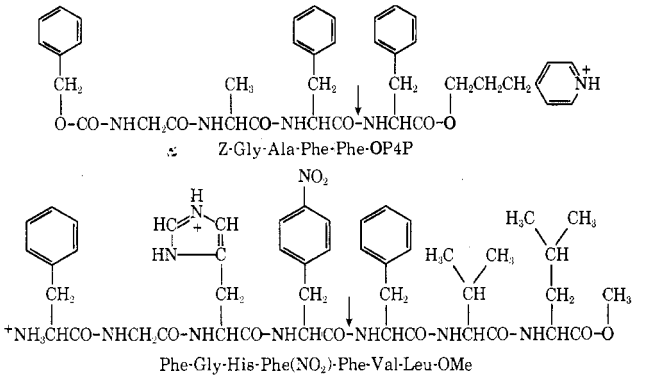


Figure 1. Synthetic substrates for pepsin.

Another series of substrates was devised in which the site of protonation is a pyridyl group as in A-Phe-Phe-OP4P.[25] For the series in which the B group of Z-His-Phe(NO_2)-Phe-B was varied, it was found that the Z-His group could be replaced by the Phe-Gly-His unit, without marked changes in the kinetic parameters for the cleavage of the Phe(NO_2)-Phe bond[26] (Figure 1).

The Secondary Specificity of Acid Proteinases

In our studies on the secondary interactions of oligopeptide substrates with pepsin, the A group of A-Phe-Phe-OP4P[19,27] and the B group of Phe-Gly-His-Phe(NO_2)-Phe-B[26,28] were varied; some of the kinetic data are given in Tables I and II. It will be evident that the structural alterations lead to striking changes in catalytic efficiency (k_{cat}) often without marked change in binding affinity (approximated by K_m). The data suggest that the extended active site of pepsin may be long enough to bind at least seven L-amino acid residues; in a fully extended conformation, such a heptapeptide would be about 25 Å long. The chain length of substrates of the type A-Phe-Phe-B is clearly not the sole factor in determining the catalytic efficiency of attack at the Phe–Phe bond. A comparison of the kinetic data in Table I for the compounds in which A = Z-Gly-Gly, Z-Gly-Ala, and Z-Gly-Pro suggests that the Z-dipeptidyl group interacts with pepsin as a unit without making a large contribution to the total binding energy in the enzyme–substrate interaction, since K_m varied between 0.1 and 0.4 mM, near the value of 0.25 mM for the dissociation constant of the complex of pepsin and the Phe-Phe unit.[21,23] On the other hand, the values of k_{cat} for these three substrates range over four orders of magnitude. These results are consistent with the view that the secondary interactions may affect catalysis by the utilization of the potential binding energy in the enzyme–substrate interaction to lower the free energy of activation in the catalytic process. This could arise by conformational change in the substrate, or the catalytic site of the enzyme, or both, leading to strain or distortion at the sensitive bond. The conformational change in the substrate may produce a transition state for which the active site has greater affinity than for either the free substrate or the products. If the active site is not a rigid structure, but can undergo conformational change in response to its interaction with the substrate, a portion of the binding energy could be used to achieve a transition state in which the active site is strained or distorted, and catalysis would be favored by the tendency of the enzyme to return to its normal state.

Table I
Kinetics of Cleavage of the Phe–Phe Bond of A-Phe-Phe-OP4P by Pepsin at pH 3.5 and 37° [19,27]

A group	k_{cat}, sec^{-1}	K_m, mM	K_{cat}/K_m
Z-	0.7	0.2	3.7
Z-Gly-	3.1	0.4	7.8
Z-$(Gly)_2$-	71.8	0.4	180
Z-$(Gly)_3$-	4.5	0.4	10.1
Z-$(Gly)_4$-	2.1	0.7	3.0
Z-Gly-Ala-	409	0.11	3720
Z-Ala-Ala-	282	0.04	7050
Z-Gly-Pro-	0.06	0.14	0.4

Table II
Kinetics of Cleavage of the Phe(NO_2)-Phe Bond of Phe-Gly-His-Phe(NO_2)-Phe-B by Pepsin at pH 4.0 and 37° [26,28]

B Group	k_{cat}, sec^{-1}	K_m, mM	K_{cat}/K_m
-OMe	0.1	0.4	0.25
-Ala-OMe	3.3	0.4	8.3
-Ala-Ala-OMe	28	0.16	175
-Ala-Phe-OMe	20	0.04	500
-Val-Leu-OMe	62	0.04	1540

The available data on the cleavage of oligopeptide substrates by other acid proteinases (cathepsin D, rennin, *Rhizopus* pepsin) are not as extensive as for gastric pepsin A, but the importance of secondary enzyme–substrate interactions is also evident, although the response to structural variation may be different.[19,28,29]

Fluorescent Probes for Secondary Interaction

Data of the kind shown in Table I indicated that the hydrophobic benzyloxycarbonyl group participates in the productive interaction of a substrate such as Z-Gly-Gly-Phe-Phe-OP4P with pepsin, although it did not appear to contribute significantly to an increase in total binding energy in the formation of the enzyme–substrate complex. It seemed possible that the amino-terminal hydrophobic group had been drawn into the extended active site of pepsin by the strong affinity of the sensitive Phe-Phe unit for the catalytic site. To test this possibility directly, we replaced the amino-terminal benzyloxycarbonyl group with either a dansyl or a mansyl group. This substitution in several of the pepsin substrates listed in Table I did not alter the relative susceptibility of the Phe–Phe bond in substrates of the type A-Phe-Phe-OP4P, although the values of both k_{cat} and K_m were found to be lower for the Dns- or Mns-peptides than for the corresponding Z-peptides.[30,31]

(24) J. Tang, *J. Biol. Chem.*, **240**, 3810 (1965).
(25) G. P. Sachdev and J. S. Fruton, *Biochemistry*, **8**, 4231 (1969).
(26) K. Medzihradszky, I. M. Voynick, H. Medzihradszky-Schweiger, and J. S. Fruton, *Biochemistry*, **9**, 1154 (1970).
(27) G. P. Sachdev and J. S. Fruton, *Biochemistry*, **9**, 4465 (1970).
(28) J. B. Ferguson, J. R. Andrews, I. M. Voynick, and J. S. Fruton, *J. Biol. Chem.*, **248**, 6701 (1973).
(29) I. M. Voynick and J. S. Fruton, *Proc. Nat. Acad. Sci. U. S.*, **68**, 257 (1971); M. N. Raymond, J. Garnier, E. Bricas, S. Cilianu, M. Blasnic, A. Chaix, and P. Lefrancier, *Biochimie*, **54**, 145 (1972); T. Oka and K. Morihara, *Arch. Biochem. Biophys.*, **156**, 552 (1973).
(30) G. P. Sachdev, M. A. Johnston, and J. S. Fruton, *Biochemistry*, **11**, 1080 (1972).
(31) G. P. Sachdev, A. D. Brownstein, and J. S. Fruton, *J. Biol. Chem.*, **248**, 6292 (1973).

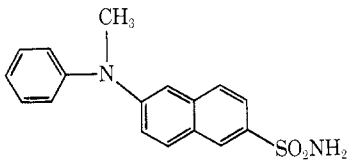
Table III
Fluorescence Measurements on the Interaction of Mansyl Compounds with Pepsin[a]

Pepsin preparation and additions	Fluorescence intensity[b]	
	Mns-NH_2	Mns-Gly-Phe-Phe-OP4P
None	0.03 (505)	0.07 (505)
Pepsin	0.20 (470)	3.1 (475)
+ pepstatin[c]	0.18 (470)	0.4 (490)
TPDM-pepsin	0.55 (465)	2.3 (475)
+ pepstatin[c]	0.57 (465)	1.8 (475)
Acetyl-pepsin	0.25 (465)	4.0 (475)
+ pepstatin[c]	1.3 (445)	3.8 (465)

[a] pH 2.35, 25°. The concentrations of mansyl compound and of pepsin (or of modified pepsin) were 10 μM. [b] Expressed as arbitrary units at maximum of corrected emission spectrum (given in nanometers in parentheses) normalized with 1.9 μM quinine sulfate in 0.1 N H_2SO_4 as the standard (1.0 at 450 nm). [c] Pepstatin concentration, 10 μM. These data are corrected for the small effect of 2% methanol (needed to introduce inhibitor) on the fluorescence intensity in the absence of pepstatin.

The dansyl group has been widely used as a probe for hydrophobic interaction because of the enhancement of its fluorescence and the shift of its emission maximum to a shorter wavelength, when this group is transferred from an aqueous environment to a solvent of low polarity or when it is bound to proteins.[32] Another widely used reagent is 6-*p*-toluidino-2-naphthalenesulfonate, but the reaction of its acid chloride with amines is attended with difficulty.[31] We therefore turned to the closely related mansyl group[33] which has been employed less extensively but offers advantages because of its greater sensitivity to changes in the polarity of its environment.[34] Thus, in a solvent of low dielectric constant (*e.g.*, dioxane), the quantum yield of the fluorescence of Mns-Phe-Phe-OP4P is about three times as great as that for Dns-Phe-Phe-OP4P.

There appear to be in pepsin at least two potential loci for binding a mansyl group. One of these sites exhibits intrinsic affinity for mansylamide (II) or the



II

mansyl group of Mns-Gly-Gly-OP4P, as judged by the enhancement of its fluorescence when mixed with pepsin in 1:1 molar proportion near pH 2.5; with dansylamide (even at 20-fold excess of protein) no change in dansyl fluorescence was noted. When a dansyl or mansyl group is located at the amino terminus of a pepsin substrate of the type A-Phe-Phe-OP4P, however, the fluorescence of the probe group is markedly enhanced when mixed with pepsin in a 1:1 molar ratio.[31] Data for some of the mansyl compounds are shown in Table III. By measurement of the change in fluorescence intensity as a function of protein concentration, at a constant concentration of Mns-Gly-Phe-Phe-OP4P, the dissociation constant (K_D) of the pepsin–ligand complex was estimated to be 0.03 mM, the value also found for K_m in the hydrolysis of this substrate by the enzyme.[31] This provides additional evidence for the view that the kinetically determined value of K_m approximates K_s and that the rate-limiting step in the catalysis is the conversion of the initial enzyme–substrate complex. It may be added that the value of K_D for Mns-NH_2 or Mns-Gly-Gly-OP4P is about 0.2 mM.

Of special importance was the finding that the large enhancement of fluorescence observed upon the addition of equimolar pepsin to a mansyl peptide such as Mns-Gly-Phe-Phe-OP4P was reduced by equimolar pepstatin to values similar to that observed with mansylamide (Table III). Pepstatin is a naturally occurring peptide which acts as a powerful competitive inhibitor of pepsin[35] and other proteinases.[28] On the other hand, the addition of pepstatin to a 1:1 mixture of pepsin and mansylamide (or Mns-Gly-Gly-OP4P) did not alter the fluorescence significantly. Since pepstatin forms a tight 1:1 complex with pepsin (K_i *ca.* 10^{-10} M), it may be inferred that the inhibitor had blocked the active site of the enzyme, thus preventing the access of the mansyl peptide, in particular its Phe-Phe unit. It may be suggested therefore that the mansyl (or dansyl) group attached to a pepsin substrate having a Phe-Phe unit is drawn into the extended active site of pepsin and that the fluorescent probe is now located in an enzymic region of low dynamic polarity that has little inherent intrinsic affinity for the mansyl group.

Further evidence for this hypothesis was provided by studies on the change in the fluorescence of mansylamide or Mns-Phe-Phe-OP4P in the presence of pepsinogen that is undergoing activation to pepsin. It had been known that the fluorescence of 6-*p*-toluidino-2-naphthalenesulfonate, closely related structurally to mansic acid, is *decreased* (about 50%) upon the autocatalytic conversion of pepsinogen to pepsin.[36] A similar result was obtained with mansylamide; with Mns-Phe-Phe-OP4P (or Dns-Gly-Pro-Phe-Phe-OP4P), however, the fluorescence of the probe group was markedly *increased* (about threefold) during the activation process.[30,31] It would appear, therefore, that the conformational changes accompanying the autocatalytic conversion of pepsinogen to pepsin led to an alteration in the binding site for mansylamide that is distinct from the alteration in the locus for the interaction with a mansyl group that is drawn in by a Phe-Phe unit of a peptide substrate.

These results suggest considerable conformational flexibility at the active site of pepsin and raise the possibility that a substrate-binding cleft in pepsin is formed through conformational changes arising from the interaction of a suitable peptide substrate or inhibitor with active site groups of the enzyme. Such a

(32) L. Stryer, *Science*, 162, 526 (1968); G. M. Edelman and W. O. McClure, *Accounts Chem. Res.*, 1, 65 (1968).

(33) R. R. Cory, R. R. Becker, R. Rosenbluth, and I. Isenberg, *J. Amer. Chem. Soc.*, 90, 1643 (1968).

(34) It should be noted that although the static interpretation of the fluorescence change in terms of transfer to a hydrophobic environment is probably correct in many cases, attention must also be given to the alternative dynamic interpretation in terms of transfer to an environment in which polar groups are present but do not undergo movement during the lifetime of the excited state.

(35) H. Umezawa, "Enzyme Inhibitors of Microbial Origin," University of Tokyo Press, Tokyo, 1972.

(36) J. L. Wang and G. M. Edelman, *J. Biol. Chem.*, 246, 1185 (1971).

cleft may be present in pepsinogen, but may be occupied by the amino-terminal portion that is removed by proteolysis during the activation process; upon conversion to pepsin, the cleft may be narrowed or closed after the release of the activation peptides and made inaccessible to the mansyl group of mansylamide. The specific interaction of a suitable peptide substrate with the catalytic site of the enzyme, in widening the cleft, thus may allow an attached mansyl group to enter an enzymic region of lower polarity. The intrinsic proteinase activity of pepsinogen[37] may therefore be a consequence of the competition between a protein substrate and the amino-terminal portion of pepsinogen for the extended active site of pepsin.

It was noted above that the enhancement of the fluorescence of mansylamide by the addition of equimolar pepsinogen is greater than with pepsin, suggesting that blockage of the active site had altered the separate binding site for mansylamide. A similar result was obtained with pepsin that had been stoichiometrically inactivated by means of the diazo reagent TPDM. Such TPDM-pepsin caused a marked increase in the fluorescence of mansylamide, as compared with untreated pepsin (Table III). This increase in fluorescence is not a consequence of tighter binding of mansylamide, since the K_D for the complex of this ligand with TPDM-pepsin is about 0.7 m*M*. It was known from earlier work[1] that the reagent specifically attacks the active site of pepsin, and binding studies[23] had shown that Phe-Phe peptides are bound much less strongly by TPDM-pepsin than by pepsin itself. It would appear, therefore, that in TPDM-pepsin the active site had not only been blocked, but that the conformation of the protein had been altered so as to decrease the polarity of the mansylamide binding site, which also accepts the mansyl group of mansyl peptides when they are excluded from the active site.

Further evidence for the considerable conformational flexibility of catalytically active pepsin was obtained in fluorescence experiments with pepsin whose tyrosyl groups had been partially acetylated. Earlier studies[18] had shown that such acetyl-pepsin exhibits a markedly increased k_{cat} for the hydrolysis of the Phe–Phe bond of Z-His-Phe-Phe-OEt, without marked change in K_m. The data on the effect of acetyl-pepsin on the fluorescence of mansylamide and of mansyl peptide substrates (Table III) indicate that the potential mansyl binding locus associated with the interaction of a Phe-Phe unit at the active site is intact, and that the other locus, which can bind the mansyl group of mansylamide and of mansyl-peptides, has become responsive to enzyme–inhibitor interactions at the active site. These results suggest that, upon acetylation, the pepsin molecule may become more flexible, possibly as a consequence of the rupture of carboxylate–phenol hydrogen bonds that are important in maintaining the three-dimensional structure of the protein.

From the foregoing evidence it would seem that pepsin has a flexible extended active site that can interact with a mansyl (or dansyl) group when it is drawn in by a Phe-Phe unit, but that it is not possible to specify a "subsite" that binds the fluorescent probe. Since the three-dimensional structure of pepsin is still unknown, more detailed speculation on this question is premature. It should be noted that attempts have been made[38] to "map" the extended active sites of proteinases by denoting the enzyme area that binds a single amino acid residue a "subsite." In such mapping, it is implicitly assumed that the active site of an enzyme has a relatively rigid structure. Although such formulations of the results of kinetic studies on a series of oligopeptide substrates lend themselves to graphical representation, they are probably oversimplifications of a more complex situation in which the conformational flexibility of the active site in response to interaction with the substrate ("induced fit") makes the mapping of the complete binding area in terms of subsites a task of doubtful validity.

The conclusions drawn for pepsin appear to be applicable to *Rhizopus* pepsin, which binds substrates of the type A-Phe-Phe-B more tightly than does pepsin. Recent fluorescence studies[39] on the interaction of *Rhizopus* pepsin with dansylamide and Dns-peptide substrates have given results entirely comparable to those summarized above for pepsin. Moreover, it has been shown that the dansyl group of Dns-peptide substrates of carboxypeptidase A is also drawn into a region of lower polarity by virtue of specific enzyme–substrate interactions at the active site.[40] Recent work has indicated that carboxypeptidase A has much more conformational flexibility at the active site than had previously been surmised from X-ray diffraction studies.[41]

The approach outlined above is also applicable to the labeling of the B portion of a pepsin substrate of the type A-Phe-Phe-B. This has been effected by the use of peptide hydrazides such as Gly-Gly-Phe-Phe-NHNH-Mns. This compound is cleaved by pepsin at the Phe–Phe bond; the fluorescence of the mansyl group is greatly enhanced upon the addition of equimolar pepsin, and is reduced to the low level observed with mansylhydrazide or mansylamide by the further addition of equimolar pepstatin.[39] It may be added that substrates such as Z-His-Phe-Phe-Aac also have been tested, and it was found that the acridine group is drawn into a region of lower polarity whereas the comparable 9-acetylaminoacridine did not appear to interact significantly with the enzyme under the experimental conditions employed.[42] The limited solubility of these compounds restricted, however, their utility for the study of the active site of pepsin.

Secondary Interactions and the Mechanism of Pepsin

Clearly, the catalytic action of many enzymes that effect the reaction of an oligomeric substrate with water, or with an alternative nucleophile in group transfer, depends importantly on the interaction of

(37) M. Bustin and A. Conway-Jacobs, *J. Biol. Chem.*, 246, 615 (1971); P. McPhie, *Biochem. Biophys. Res. Commun.*, 56, 789 (1974).

(38) A. Berger and I. Schechter, *Phil. Trans. Roy. Soc. London, Ser. B*, 257, 249 (1970).

(39) Unpublished results of G. P. Sachdev and A. D. Brownstein in this laboratory.

(40) S. Latt, D. S. Auld, and B. L. Vallee, *Proc. Nat. Acad. Sci. U. S.*, 67, 1383 (1070).

(41) W. N. Lipscomb, *Proc. Nat. Acad. Sci. U. S.*, 70, 3797 (1973).

(42) G. P. Sachdev, A. D. Brownstein, and J. S. Fruton, *J. Biol. Chem.*, 249, 420 (1974).

the substrate with regions of the enzyme relatively distant from the catalytic site, with the virtually simultaneous formation or scission of several noncovalent bonds (multiple cooperative interaction). Evidence for this conclusion is available not only for pepsin and pepsin-like enzymes, but also for other proteinases, notably papain,[38] elastase,[43] and chymotrypsin.[44] Moreover, enzymes acting on oligosaccharides (lysozyme,[45] α-amylase[46]) are known to have extended active sites. It is noteworthy that all these enzymes catalyze both hydrolytic reactions and transpeptidation or transglycosylation reactions[47] and it appears likely that secondary enzyme–substrate interactions may play a significant role in the catalytic mechanisms that are involved. In particular, transfer reactions such as AB + HX $\rightleftarrows$ AX + BH require an ordered release of the A and B portions of AB, and significant differences in the energy of the interactions of A and B portions in a series of substrates for a given enzyme may have considerable effect on the kinetics of that enzyme. In the special case of pepsin, transpeptidation experiments have suggested that substrates such as Ac-Phe-Tyr are cleaved with the apparent prior release of the Ac-Phe portion and that the kinetic equivalent of an "imino-enzyme" (E-Tyr) can react with a suitable carboxylic acid to form RCO-Tyr. Several hypotheses have been offered[8,9] to rationalize the transformation of the presumed tetrahedral intermediate I to an imino-enzyme, with the release of the acyl portion of the substrate. The status of these hypotheses is uncertain, however, since neither Ac-Phe-Tyr-NH_2 nor Ac-Phe-Phe-OEt gives transpeptidation products, raising doubt about the occurrence of a covalently bound E-Tyr intermediate.[48]

Clearly, the further study of the mechanism of pepsin action requires closer study with substrates of the type A-Phe-Phe-OP4P and Phe-Gly-His-Phe(NO_2)-Phe-B, where the A and B groups contain amino acid units that enhance the catalytic efficiency of the enzyme. Moreover, an examination of the action of pepsin on comparable ester substrates, where the sensitive Phe-Phe unit has been replaced by Phe-Pla, is needed. It will be of interest to compare the results with those for carboxypeptidase A, whose mechanism appears to resemble that of pepsin in several respects.[49]

The research of our laboratory reported in this article was aided by grants from the National Institutes of Health (GM-18172 and AM-15682) and from the National Science Foundation (GB-37871). I also thank Professor Lubert Stryer for many helpful discussions and for his valuable comments on an early draft of this article.

(43) R. C. Thompson and E. R. Blout, *Biochemistry*, **12**, 57 (1973); D. Atlas and A. Berger, *ibid.*, **12**, 2573 (1973).
(44) W. K. Baumann, S. A. Bizzozero, and H. Dutler, *Eur. J. Biochem.*, **39**, 381 (1973).
(45) D. M. Chapman and N. Sharon, *Science*, **165**, 454 (1969).
(46) J. F. Robyt and D. French, *J. Biol. Chem.*, **245**, 3917 (1970).
(47) J. S. Fruton, *Harvey Lect.*, **51**, 64 (1957).
(48) M. S. Silver and M. Stoddard, *Biochemistry*, **11**, 191 (1973).
(49) E. T. Kaiser and B. L. Kaiser, *Accounts Chem. Res.*, **5**, 219 (1972).

Equilibrium Structure and Molecular Motion in Liquids

David Chandler

School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801

Received March 27, 1974

Much progress has been made recently in the theory of liquids.[1] The principal physical concept associated with this progress originated with the work of van der Waals long ago. It is the idea that, for a dense fluid, the repulsive forces (which are nearly hard-core interactions) dominate the liquid structure. This means that the *shape* of molecules determines the intermolecular correlations. Attractive forces, dipole–dipole interactions, and other slowly varying forces play a minor role. As a result, if a dense liquid is composed of spherical (or nearly spherical) molecules, the intermolecular structure should be very similar to that of a fluid made up of hard spheres.

Most of the modern theories of liquids have focussed attention on this concept, and calculations have shown that the idea is quantitatively accurate for many liquids.[1-9] Further, it has been shown by Longuet-Higgins and Widom[7] and by others[1,9] that liquids freeze when the density becomes high enough that *steric effects* (not attractive forces) lock the molecules into a structure for which particle diffu-

Professor Chandler's research interests include microscopic theory of the static and dynamic structure of simple and molecular liquids, ionic solutions, and phase transitions. He was born in Brooklyn in 1944. He received the B.S. degree from Massachusetts Institute of Technology and the Ph.D. from Harvard University (in 1969), after which he spent a postdoctoral year at University of California, San Diego. Since 1970 he has been on the faculty of the University of Illinois at Urbana–Champaign. He is an Alfred P. Sloan Foundation Research Fellow.

(1) For a recent review, see J. A. Barker and D. Henderson, *Annu. Rev. Phys. Chem.*, **23**, 439 (1972).
(2) D. Chandler and J. D. Weeks, *Phys. Rev. Lett.*, **25**, 149 (1970).
(3) J. D. Weeks, D. Chandler, and H. C. Andersen, *J. Chem. Phys.*, **54**, 5237 (1971); **55**, 5421 (1971).
(4) H. C. Andersen, J. D. Weeks, and D. Chandler, *Phys. Rev. A*, **4**, 1597 (1971).
(5) L. Verlet and J.-J. Weis, *Phys. Rev. A*, **5**, 939 (1972).
(6) J. A. Barker and D. Henderson, *Phys. Rev. A*, **4**, 806 (1971).
(7) H. C. Longuet-Higgins and B. Widom, *Mol. Phys.*, **8**, 549 (1964); B. Widom, *Science*, **157**, 375 (1967).
(8) J. A. Barker and D. Henderson, *Accounts Chem. Res.*, **4**, 303 (1971); J. A. Barker and D. Henderson, *J. Chem. Phys.*, **47**, 4714 (1967).
(9) L. Verlet and J.-J. Weis, *Mol. Phys.*, **24**, 1013 (1972).
(10) J. P. Hansen and L. Verlet, *Phys. Rev.*, **184**, 151 (1969); D. Henderson and J. A. Barker, *Mol. Phys.*, **14**, 587 (1968); J.-J. Weis, preprint; R. K. Crawford, *J. Chem. Phys.*, **60**, 2169 (1974).