Structural Basis of the Action of Thermolysin and Related Zinc Peptidases

BRIAN W. MATTHEWS

Institute of Molecular Biology and Department of Physics, University of Oregon, Eugene, Oregon 97403

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Recent crystallographic and other studies have suggested a detailed mechanism of action for thermolysin, carboxypeptidase A, and related zinc endopeptidases. These studies also have implications for the energetics and kinetics of enzyme–inhibitor interaction and, in addition, illustrate the potential of rational drug design. This report is intended as a brief overview of these recent developments.

Thermolysin (EC 3.4.24.4) is a thermostable calcium-binding zinc endopeptidase of molecular weight 34 600 isolated from the thermophile *Bacillus thermoproteolyticus*.[1] The amino acid sequence is known,[2] and the three-dimensional structure has been determined and refined at high resolution.[3-5] Parallel sequence[6] and structural[7] studies show that the neutral proteases from *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus amyloliquefaciens*, and *Bacillus cereus* are closely related to thermolysin.

It is less clear whether or not there are structural relationships between thermolysin and the zinc-containing peptidases from higher organisms. Such proteins include carboxypeptidase A, the angiotensin converting enzyme, enkephalinase, and collagenase. With the exception of carboxypeptidase A, very little is known of the three-dimensional structures of these physiologically significant enzymes. Recent evidence, however, suggests that the active sites of some, if not all, of these metallopeptidases have features in common.[8]

Structure of Thermolysin

The structure of thermolysin is bilobal, with a pronounced active-site cleft being formed at the junction of the two domains[3] (Figure 1). The enzyme binds four calcium ions that are necessary for thermal stability.[1,3,9] It appears that the calcium ions increase the intrinsic thermostability of the protein and protect surface loops against autolysis;[10,11] however, the detailed mechanism of thermal stabilization has not been elucidated. The relative binding affinities of the four calcium ions is a matter of debate.[10-13]

Thermolysin can be proteolytically cleaved into distinct fragments.[11,14] The sites of proteolytic cleavage correspond to solvent-exposed surface loops that are observed to have high mobility in the crystal structure.[15]

Inhibitor Binding

The modes of binding of a number of thermolysin inhibitors have been determined crystallographically by diffusing the inhibitor into pregrown crystals of the enzyme[16-38] (Table I; Figure 2). In early experi-

Brian W. Matthews was born in Australia and received his B.Sc., Ph.D., and D.Sc. from the University of Adelaide. He was a member of staff at the Medical Research Council Laboratory of Molecular Biology, Cambridge, England (with D. M. Blow) and a Visiting Associate at the National Institutes of Health (with D. R. Davies). In 1969 he joined the faculty of the University of Oregon, where he is Professor of Physics and Member of the Institute of Molecular Biology. His research interests include DNA-protein interaction and the structure, function, stability, and evolution of proteins.

(1) Endo, S. *J. Ferment. Technol.* **1962**, *40*, 346.
(2) Titani, K.; Hermodson, M. A.; Ericsson, L. H.; Walsh, K. A.; Neurath, H. *Nature (London), New Biol.* **1972**, *238*, 35.
(3) Matthews, B. W.; Jansonius, J. N.; Colman, P. M.; Schoenborn, B. P.; Dupourque, D. *Nature (London), New Biol.* **1972**, *238*, 37. Matthews, B. W.; Colman, P. M.; Jansonius, J. N.; Titani, K.; Walsh, K. A.; Neurath, H. *Nature (London), New Biol.* **1972**, *238*, 41. Colman, P. M.; Jansonius, J. N.; Matthews, B. W. *J. Mol. Biol.* **1972**, *70*, 701.
(4) Matthews, B. W.; Weaver, L. H.; Kester, W. R. *J. Biol. Chem.* **1974**, *249*, 8030.
(5) Holmes, M. A.; Matthews, B. W. *J. Mol. Biol.* **1982**, *160*, 623.
(6) Yang, M. Y.; Ferrari, E.; Henner, D. J. *J. Bacteriol.* **1984**, *160*, 15. Takagi, M.; Imanaka, T.; Aiba, S. *J. Bacteriol.* **1985**, *163*, 824. Vasantha, N.; Thompson, L. D.; Rhodes, C.; Banner, C.; Nagle, J.; Filpula, D. *J. Bacteriol.* **1984**, *159*, 811. Sidler, W.; Niederer, E.; Suter, F.; Zuber, H. *Biol. Chem.* Hoppe-*Seyler* **1986**, *367*, 643.
(7) Pauptit, R. A.; Karlsson, R.; Picot, D.; Jenkins, J. A.; Niklaus-Reimer, A. S.; Jansonius, J. N. *J. Mol. Biol.* **1988**, *199*, 525.
(8) Powers, J. C.; Harper, J. W. *Am. Rev.* Respir. *Dis.* **1986**, *134*, 1092. Benchetrit, T.; Bissery, V.; Mornon, J. P.; Devault, A.; Crine, P.; Roques, B. P. *Biochemistry* **1988**, *27*, 592. Fricker, L. D.; Evans, C. J.; Esch, F. S.; Herbert, E. *Nature (London)* **1986**, *323*, 461. Bond, J.; Beynon, R. J. *Int. J. Biochem.* **1985**, *17*, 565. Mookhtiar, K. A.; Wang, F.; Van Wart, H. E. *Arch. Biochem. Biophys.* **1986**, *246*, 645.
(9) Feder, J.; Garrett, L. R.; Wildi, B. S. *Biochemistry* **1971**, *10*, 4552.
(10) Roche, R. S.; Voordouw, G. *CRC Crit. Rev. Biochem.* **1978**, *6*, 1. Corbett, J. T.; Roche, R. S. *Biopolymers* **1983**, *22*, 101.
(11) Fassina, G.; Vita, C.; Dalzoppo, D.; Zamai, M.; Zambonin, M.; Fontana, A. *Eur. J. Biochem.* **1986**, *156*, 221.
(12) Voordouw, G.; Roche, R. S. *Biochemistry* **1974**, *13*, 5017. Dahlquist, F. W.; Long, J. W.; Bigbee, W. L. *Biochemistry* **1976**, *15*, 1103.
(13) Weaver, L. H.; Kester, W. R.; Ten Eyck, L. F.; Matthews, B. W. *Symposium on Enzymes and Proteins from Thermophilic Microorganisms*, Zuber, H., Ed.; *Experientia Suppl.* **1976**, *26*, 31.
(14) Vita, C.; Dalzoppo, D.; Fontana, A. *Biochemistry* **1985**, *24*, 1798.
(15) Fontana, A.; Fassina, G.; Vita, C.; Dalzoppo, D.; Zamai, M.; Zambonin, M. *Biochemistry* **1986**, *25*, 1847.
(16) Kester, W. R.; Matthews, B. W. *Biochemistry* **1977**, *16*, 2506.
(17) Holmquist, B.; Vallee, B. L. *J. Biol. Chem.* **1974**, *249*, 4601.
(18) Feder, J.; Auferheide, N.; Wildi, B. S. In Enzymes *and Proteins from Thermophilic Microorganisms*; Zuber, H., Ed.; Birkhauser Verlag: Basel, Switzerland, 1976; p 31.
(19) Holden, H. M.; Matthews, B. W. *J. Biol. Chem.* **1987**, *26*, 8553.
(20) Suda, H.; Aoyagi, T.; Takeuchi, T.; Umezawa, H. *J. Antibiot.* **1973**, *26*, 621. Komiyama, T.; Suda, H.; Aoyagi, T.; Takeuchi, T.; Umezawa, H.; Fujimoto, K.; Umezawa, S. *Arch. Biochem. Biophys.* **1975**, *171*, 727.
(21) Weaver, L. H.; Kester, W. R.; Matthews, B. W. *J. Mol. Biol.* **1977**, *114*, 119.
(22) Tronrud, D. E.; Monzingo, A. F.; Matthews, B. W. *Eur. J. Biochem.* **1986**, *157*, 261.
(23) Kam, C.-M.; Nishino, N.; Powers, J. C. *Biochemistry* **1979**, *18*, 3032.
(24) Bartlett, P. A.; Marlowe, C. K. *Biochemistry* **1987**, *26*, 8553.
(25) Holden, H. M.; Tronrud, D. E.; Monzingo, A. F.; Weaver, L. H.; Matthews, B. W. *Biochemistry* **1987**, *26*, 8542.
(26) Bartlett, P. A.; Marlowe, C. K. Science (*Washington, D.C.*) **1987**, *235*, 569.
(27) Tronrud, D. E.; Holden, H. M.; Matthews, B. W. *Science (Washington, D.C.)* **1987**, *235*, 571.
(28) Nishino, N.; Powers, J. C. *Biochemistry* **1979**, *18*, 4340.
(29) Monzingo, A. F.; Matthews, B. W. *Biochemistry* **1982**, *21*, 3390.
(30) Byers, L. D.; Wolfenden, R. *J. Biol. Chem.* **1972**, *247*, 606.
(31) Bolognesi, M. C.; Matthews, B. W. *J. Biol. Chem.* **1979**, *254*, 634.
(32) Maycock, A. L.; DeSousa, D. M.; Payne, L. G.; ten Broeke, J.; Wu, M. T.; Patchett, A. A. *Biochem. Biophys. Res. Commun.* **1981**, *102*, 963.
(33) Monzingo, A. F.; Matthews, B. W. *Biochemistry* **1984**, *23*, 5724.
(34) Hangauer, D.; Ondeyka, D.; Bull, H.; Thornberry, N.; Ulm, E.; LaMont, B.; Holden, H.; Weaver, L.; Tronrud, D.; Matthews, B., in preparation.
(35) Nishino, N.; Powers, J. C. *Biochemistry* **1978**, *17*, 2846.
(36) Holmes, M. A.; Matthews, B. W. *Biochemistry* **1981**, *20*, 6912.

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Table I
Some Inhibitors of Thermolysin Analyzed Crystallographically

inhibitor	abbrev name	K_i, μM	crystallographic resol, Å	*R* value, %	ref
β-(phenylpropionyl)-L-phenylalanine	βPPP	1600	2.3		16, 17
carbobenzoxy-L-phenylalanine	Z-Phe	510	2.3		16, 18
L-valyl-L-tryptophan	Val-Trp		1.7	17.3	19
N-((α-L-rhamnopyranosyloxy)hydroxyphosphinyl)-L-Leu-L-Trp	phosphoramidon	0.028	2.3	17.4	20–22
N-phosphoryl-L-leucinamide	P-Leu-NH_2	21.3	1.6	17.9	22, 23
N-[[1-[(phenylmethoxycarbonyl)amino]-2-phenylethyl]methoxyphosphinyl]-L-Leu-L-Ala-methyl ester (Cbz-*Phe*^{P}-L-Leu-L-Ala)	ZF^{P}LA	0.000068	1.7	17.0	24, 25
Cbz-*Gly*^{P}-L-Leu-L-Leu	ZG^{P}LL	0.0091	1.6	17.7	25, 26
Cbz-*Gly*^{P}(O)-L-Leu-L-Leu	ZG^P(O)LL	9.0	1.6	17.1	26, 27
(2-benzyl-3-mercaptopropanoyl)-L-alanylglycinamide	BAG	0.75	1.9	18.4	28, 29
L-benzylsuccinic acid	BZSA	3800	2.3		30, 31
N-(1-carboxy-3-phenylpropyl)-L-Leu-L-Trp	CLT	0.05	1.9	17.1	32, 33
N-[1-carboxy-3-((*N*′-carbobenzoxy-L-phenylalanyl)amino)propyl]-L-Leu-L-Trp	CCPALT[a]	0.011	1.6[a]	18.0[a]	34
L-leucine hydroxamic acid	Leu-NHOH	190	2.3		35, 36
HONH-(benzylmalonyl)-L-Ala-Gly-*p*-nitroanilide	HONH-BAGN	0.43	2.3		35, 36
N-(chloroacetyl)-DL-*N*-hydroxyleucine methyl ester	CHME	covalent	2.3		37, 38

[a] The X-ray analysis does not correspond to the intact inhibitor.[34]

Table II
Structural Correspondence between Thermolysin Inhibitors and the Presumed Transition State[a]

inhibitor	dist between corresponding atoms, Å: O1	O2	N	ref
Val-Trp			0.5	19
phosphoramidon	0.7	0.6	1.1	22
P-Leu-NH_2	0.3	0.5	0.2	22
ZF^{P}LA	0.1	0.1	0.1	25
ZG^{P}LL	0.2	0.6	0.1	25
ZG^P(O)LL	0.4	0.5	0.1	27
CLT	0.8	0.5	0.5	33
Leu-NHOH	0.4	0.7		36
HONH-BAGN	0.9	0.5		36

[a] The table gives the distance between the two oxygens and the nitrogen of the presumed transition state[25] for cleavage of peptides and structurally analogous atoms as observed in different thermolysin–inhibitor complexes.

ments[16,21,31,36] the data were collected by precession photography. This required perhaps 20 good crystals and limited the resolution to 2.3 Å. Such studies yield a coordinate accuracy of about 0.3–0.4 Å. In more recent studies[19,22,25,27,29,33,38] the use of oscillation photography[39] has permitted data to about 1.7-Å resolution to be measured from one or two crystals. These high-resolution data sets, coupled with crystallographic refinement,[5,40] provide coordinates that have a root-mean-square positional uncertainty of about 0.15 Å.

Mechanism of Action

Crystallographic analyses of enzyme–inhibitor complexes are routinely used to suggest how enzymes interact with their cognate substrates. Suitably chosen inhibitors may provide structural models for various stages in catalysis, including the Michaelis complex, transition states, and products. Such studies can suggest, or at least severely proscribe, possible mechanisms of catalysis. The details of putative catalytic pathways can be explored in detail by model building and computer graphics. Such has been done for thermolysin.[41] It must be emphasized, however, that conventional crystallographic techniques require long-lived inhibitor complexes that are stable for at least a week. Such complexes may provide *structural models* for different stages in catalysis, but they are obviously not true intermediates and, in the absence of supporting data, must be interpreted with caution.

The mechanism of action of thermolysin that has emerged from an extended series of structural studies is summarized in Figure 3.[16,36,41] In native thermolysin the zinc ion has approximate tetrahedral coordination, with three ligands provided by the protein (His 142, His 146, and Glu 166) and the fourth provided by a water molecule. The incoming substrate is presumed to displace this solvent molecule (still bound to the zinc) toward Glu 143. Model building suggests that the Michaelis complex is formed when the carbonyl oxygen of the scissile bond is about 4 Å from the zinc.[41] In this complex (not shown in Figure 3) the carbonyl oxygen is presumed to form hydrogen bonds with both His 231 and the zinc-bound water molecule.

Early structural studies suggested that the thermolysin-catalyzed cleavage of peptide bonds proceeds via a general-base-type mechanism with the attack of a water molecule or hydroxide ion on the carbonyl carbon of the scissile peptide bond.[16] The alternative "anhydride" mechanism was disfavored because stereochemical restrictions appeared to exclude the possibility of direct nucleophilic attack of the carbonyl carbon by Glu 143. Independent support for the "general base" rather than the "anhydride" pathway has come from studies of thermolysin-catalyzed peptide bond synthesis, including the monitoring of ^{18}O exchange.[42] The general-base mechanism is also consistent with the pH and temperature dependence of thermolysin catalysis.[43]

Detailed model building[41] suggests that the incoming substrate optimizes its interactions in the S_2, S_1, S_1', and

(37) Rasnick, D.; Powers, J. C. *Biochemistry* **1978**, *17*, 4363.
(38) Holmes, M. A.; Tronrud, D. E.; Matthews, B. W. *Biochemistry* **1983**, *22*, 236.
(39) Rossmann, M. G. *J. Appl. Crystallogr.* **1979**, *12*, 225. Schmid, M. F.; Weaver, L. H.; Holmes, M. A.; Grutter, M. G.; Ohlendorf, D. H.; Reynolds, R. A.; Remington, S. J.; Matthews, B. W. *Acta Crystallogr., Sect. A.* **1981**, *A37*, 701.
(40) Tronrud, D. E.; Ten Eyck, L. F.; Matthews, B. W. *Acta Crystallogr., Sect. A.* **1987**, *A34*, 489.
(41) Hangauer, D. G.; Monzingo, A. F.; Matthews, B. W. *Biochemistry* **1984**, *23*, 5730.
(42) Oyama, K.; Kihara, K.; Nonaka, Y. *J. Chem. Soc., Perkin Trans. 2* **1981**, 356. Wayne, S. I.; Fruton, J. S. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 3241. Antonov, V. K.; Ginodman, L. M.; Rumsh, L. D.; Kapitannikov, Y. V.; Barshevskaya, T. N.; Yavashev, L. P.; Gurova, A. G.; Volkova, L. I. *Eur. J. Biochem.* **1981**, *117*, 195.
(43) Kunugi, S.; Hirohara, H.; Ise, N. *Eur. J. Biochem.* **1982**, *124*, 157.

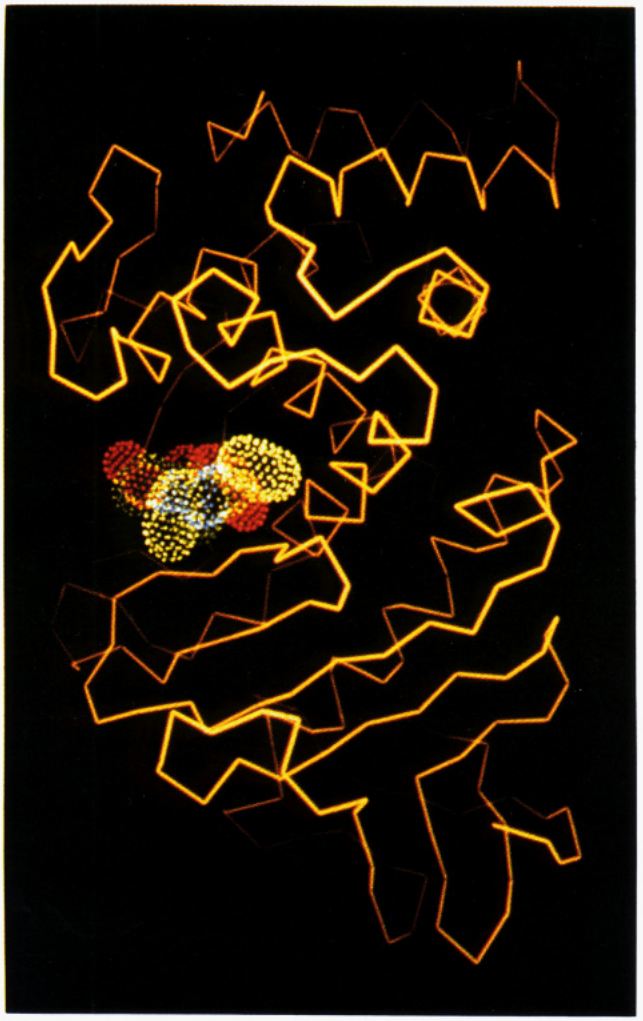

Figure 1. Schematic diagram showing the configuration of the α-carbon backbone of thermolysin. The dotted surface outlines an inhibitor bound within the active-site cleft. (Figure courtesy of Drs. H. M. Holden and D. E. Tronrud.)

S_2' subsites[44] by driving the zinc-bound water molecule toward Glu 143. The nucleophilicity of this water molecule could be enhanced by having both protons hydrogen bonded to Glu 143, and, at the same time, having the oxygen liganded to the zinc ion as suggested in Figure 3a. This tripartite interaction would leave the remaining lone pair directed toward the carbonyl carbon of the substrate and aligned for nucleophilic attack. Under the combined influence of the metal ion and the glutamate, the water attacks the carbonyl carbon to form the pentacoordinate[36,45] intermediate shown in Figure 3b. The proton accepted by Glu 143 is then immediately transferred to the leaving nitrogen (Figure 3c).

As shown in Figures 4 and 5 there are several tight-binding inhibitors of thermolysin that provide structural analogues for the presumed tetrahedral intermediate.[22,25,27,33] These inhibitors illustrate in some detail the presumed enzyme–substrate interactions. One of the oxygens of the hydrated peptide interacts with the zinc ion and, in addition, is stabilized by hydrogen bonds from both His 231 and Tyr 157 (Figure 3b). This

(44) The identification of subsites is as introduced by: Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157.

(45) Argos, P.; Garavito, R. M.; Eventoff, W.; Rossmann, M. G.; Branden, C. I. *J. Mol. Biol.* **1978**, *126*, 141.

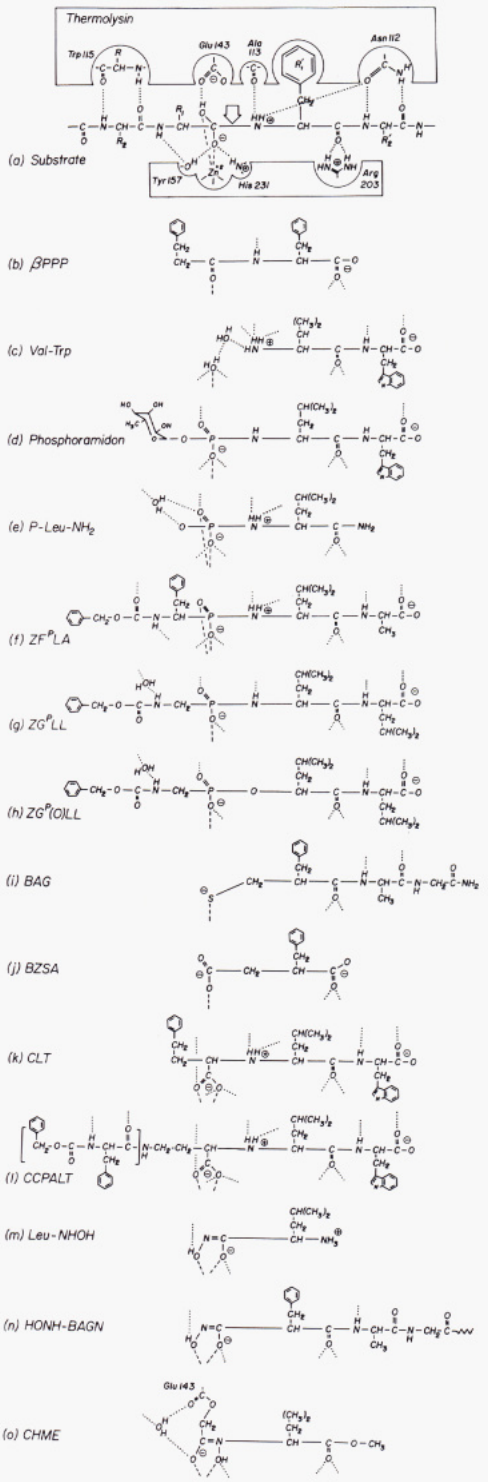

Figure 2. Schematic diagram showing the interactions between thermolysin and a number of inhibitors that have been analyzed crystallographically. Presumed hydrogen bonds are shown as dotted lines and interactions with the zinc as broken lines. The interactions shown at the top of the figure are those that are *assumed* to occur for the tetrahedral transition state of an extended polypeptide substrate. The bond to be cleaved is indicated by an arrowhead. Interactions shown for all the inhibitors presented in the figure are substantiated by crystallographic analyses, with the exception of CCPALT. In this case the compound shown was designed and found to be a very tight-binding inhibitor, but crystallographic analysis revealed no electron density for the part of the inhibitor enclosed in parentheses.[34] This figure is to be read in conjunction with Table I, which provides references and additional information.

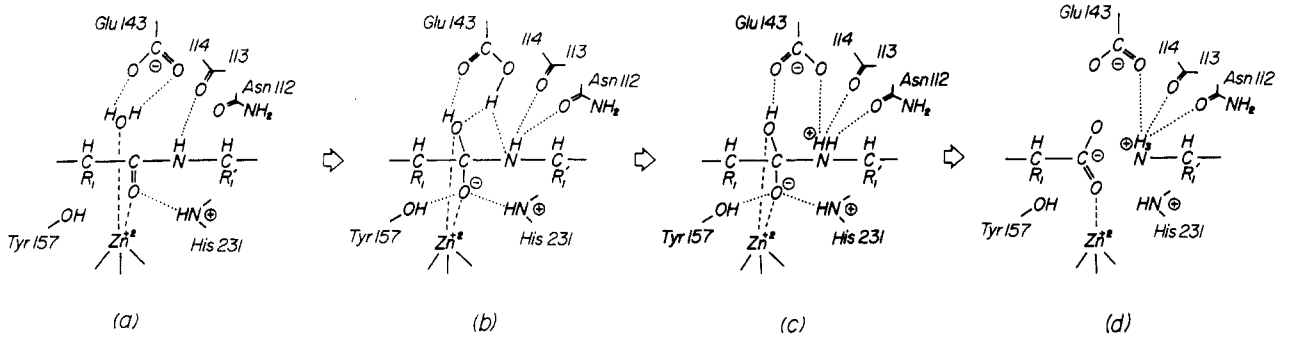

Figure 3. Proposed mechanism for the thermolysin-catalyzed cleavage of peptides (after ref 41).

pair of hydrogen bonds is analogous to the "oxyanion hole" of the serine proteases.[46] The second oxygen of the hydrated peptide is also stabilized by interaction with the zinc as well as a strong hydrogen bond with Glu 143. The side-chain oxygen of Asn 112 and the backbone carbonyl of Ala 113 accept hydrogen bonds from the doubly protonated tetrahedral nitrogen of the scissile bond (Figure 3c). These interactions are best exemplified by carbobenzoxy-*Phe*^{P}-Leu-Ala (ZF^{P}LA) (Figures 4, 5b),[24,25] an extremely tight-binding inhibitor of thermolysin (K_i = 0.068 nm)[24] which is presumed to be a direct analogue of the tetrahedral intermediate (see also Figure 2a,f). The collapse of this intermediate (Figure 3c) to products (Figure 3d) is presumed to be facilitated by a second proton transfer via Glu 143 (not shown in Figure 3). In this case the proton is accepted from the hydrated peptide and shuttled to the leaving nitrogen.[41] The recent determination of the complex of Val-Trp with thermolysin[19] provides a direct model for the binding of the amine product of peptide hydrolysis and suggests that the newly formed amino group interacts with the enzyme as illustrated in Figure 3d. The mode of interaction of the acidic product is less certain.

The early suggestion that His 231 might act as a proton donor[16,45,47] now seems unlikely. However, the evidence that His 231 must be in its *protonated* state for catalysis[45] is consistent with the idea that this residue helps stabilize the transition state by *donating* a hydrogen bond to the hydrated peptide (Figure 3b).

Thermolysin and Carboxypeptidase A

Although the amino acid sequences and the overall tertiary structures of thermolysin and carboxypeptidase A[48] are unrelated, the active sites of these two metallopeptidases have elements in common.[45,50] Superposition of one active site on the other shows that the position of Glu 270 of carboxypeptidase A relative to the zinc and to a bound dipeptide corresponds closely to that of Glu 143 of thermolysin. This close correspondence suggested that the chemical and stereochemical combination of the zinc ion plus the acid group (Glu 143 of thermolysin; Glu 270 of carboxypeptidase) was vital to catalysis.[50] In contrast, it was also found that His 231 of thermolysin and Tyr 248 of carboxypeptidase A, the two groups presumed (at that time) to be proton donors, did *not* occupy similar positions. This structural mismatch led to the conclusion that there was no absolute requirement for the involvement of a histidine or a tyrosine in hydrolysis catalyzed by the zinc neutral proteases.[50]

The above differences between the active sites of thermolysin and carboxypeptidase can be reconciled by postulating[33] that the mechanism of peptide cleavage by carboxypeptidase is analogous to that proposed in Figure 3 for thermolysin. In the proposed carboxypeptidase A mechanism[33] (Figure 6), Glu 270 is presumed to act in conjunction with the zinc to promote the attack of a water molecule on the carbonyl carbon (Figure 6b). Glu 270 is then assumed to shuttle two protons successively to the leaving nitrogen (Figure 6c,d), analogous to the mechanistic scenario that has been described for thermolysin. The proposal that Glu 270 of carboxypeptidase A acts as a general base has been considered for a long time[48,51] and is supported by evidence from kinetic isotope effects[51] and from the analysis of intermediates by cryokinetic and cryospectroscopic techniques[52] (but see also ref 53). The additional proposal that Glu 270 rather than Tyr 248 acts as proton donor was not favored previously[49] but has been supported by the demonstration that Tyr 248 of carboxypeptidase A can be replaced with phenylalanine with little effect on catalytic activity.[54] The proposed mechanism[33] has recently been supported by model studies[55] and has been adopted by Lipscomb and coworkers[56] to rationalize the binding of substrate analogues to the crystalline enzyme.

The similar mechanisms proposed for thermolysin (Figure 3) and carboxypeptidase A (Figure 6) are com-

(46) Robertus, J. D.; Kraut, J.; Alden, R. A.; Birktoft, J. J. *Biochemistry* **1972**, *11*, 4293.

(47) Pangburn, M. K.; Walsh, K. A. *Biochemistry* **1975**, *14*, 4050.

(48) Lipscomb, W. N.; Hartsuck, J. A.; Reeke, G. N., Jr.; Quiocho, F. A.; Bethge, P. H.; Ludwig, M. L.; Steitz, T. A.; Muirhead, H.; Coppola, J. C. *Brookhaven Symp. Quant. Biol.* **1968**, *21*, 24. Lipscomb, W. N. *Acc. Chem. Res.* **1982**, *15*, 232. Lipscomb, W. N. *Annu. Rev. Biochem.* **1983**, *52*, 17.

(49) Rees, D. C.; Lipscomb, W. N. *J. Mol. Biol.* **1982**, *160*, 475.

(50) Kester, W. R.; Matthews, B. W. *J. Biol. Chem.* **1977**, *252*, 7704.

(51) Breslow, R.; Wernick, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1303. Breslow, R.; Chin, J.; Hilvert, D.; Trainor, G. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 4585.

(52) Geoghegan, K. F.; Holmquist, B.; Spilburg, C. A.; Vallee, B. L. *Biochemistry* **1983**, *22*, 1847. Galdes, A.; Auld, D. S.; Vallee, B. L. *Biochemistry* **1986**, *25*, 646.

(53) There is good evidence that certain conjugated ester substrates of carboxypeptidase A are cleaved via the anhydride pathway. All examples of this type reported to date are closely related to *O*-(*trans*-*p*-chlorocinnamoyl)-L-phenyllactate. Makinen, M. W.; Kuo, L. C.; Dymowski, J.; Jaffer, S. *J. Biol. Chem.* **1979**, *254*, 356. Kuo, L. C.; Fukuyama, J. M.; Makinen, M. W. *J. Mol. Biol.* **1983**, *163*, 63. Kuo, L. C.; Makinen, M. W. *J. Am. Chem. Soc.* **1985**, *107*, 5255. Sander, M. E.; Witzel, H. *Biochem. Biophys. Res. Commun.* **1985**, *132*, 681. Suh, J.; Hong, S.-B.; Chung, S. *J. Biol. Chem.* **1986**, *261*, 7112.

(54) Gardell, S. J.; Craik, C. S.; Hilvert, D.; Urdea, M. S.; Rutter, W. J. *Nature (London)* **1985**, *317*, 551.

(55) Breslow, R.; Schepartz, A. *Chem. Lett.* **1987**, 1. Schepartz, A.; Breslow, R. *J. Am. Chem. Soc.* **1987**, *109*, 1814.

(56) Christianson, D. W.; David, P. R.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 1512.

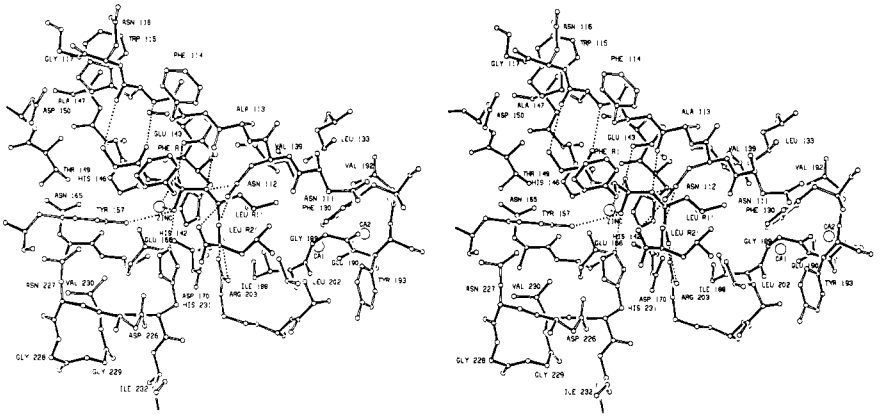

Figure 4. Stereodrawing showing the binding of ZF^{P}LA to thermolysin. Presumed hydrogen bonds and interactions with the zinc are indicated by hydrogen bonds (from ref 25).

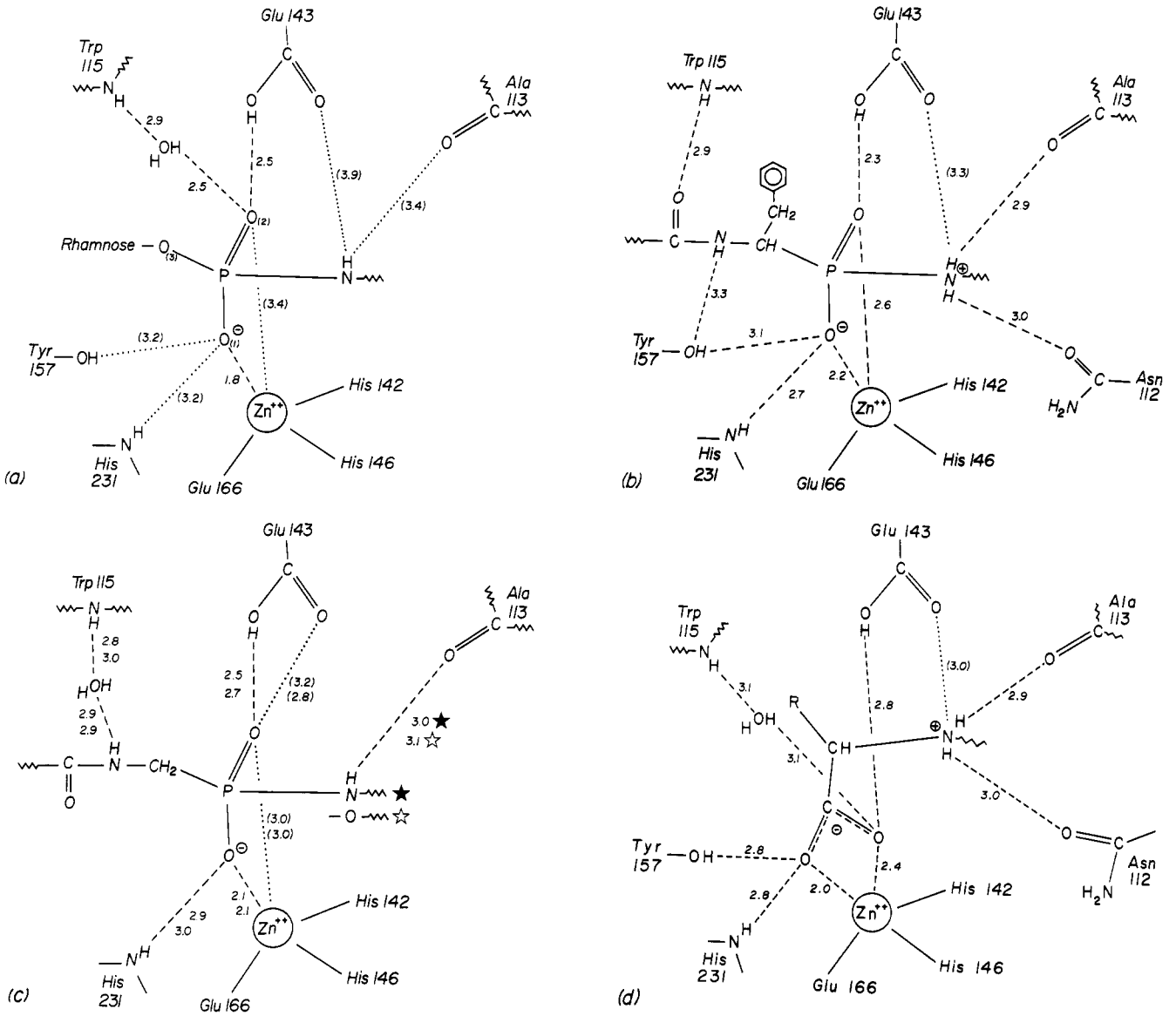

Figure 5. Detailed interactions of a number of tight-binding thermolysin inhibitors, each of which has at least partial resemblance to the presumed transition state. Presumed hydrogen bonds and zinc-ligand interactions are indicated by broken lines with distances in angstroms. Other close approaches are shown as dotted lines with distances in parentheses: (a) phosphoramidon;[21,22] (b) ZF^{P}LA;[25] (c) ZG^{P}LL (distances above) and ZG^{P}(O)LL (distances below);[27] (d) CLT.[33] Additional details for these inhibitors are included in Table I and Figure 2.

patible with the stereochemical relationships between the respective active sites. The groups of the respective enzymes that are presumed to play common roles (i.e., the zinc ions and the glutamates) are structurally superimposable. Groups that are thought to play secondary roles in substrate binding and alignment do not

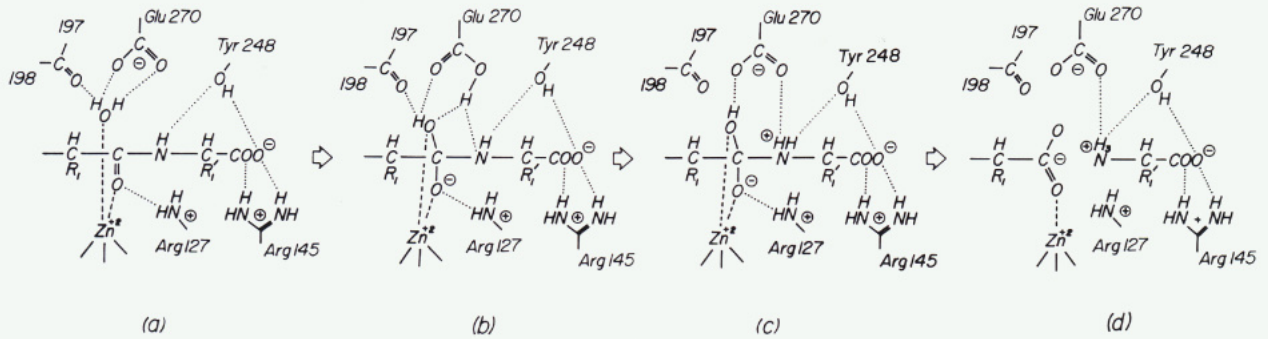

Figure 6. Proposed mechanism for the carboxypeptidase A catalyzed hydrolysis of peptide bonds, based on its presumed analogy with the mechanism for thermolysin outlined in Figure 3. The central features of the mechanism, including the attack of the water molecule on the carbonyl carbon of the scissile bond, the acceptance of a proton from the attacking water by Glu 270, and the transfer of this proton to the leaving nitrogen, are as proposed by Monzingo and Matthews.[33] The interactions shown for Arg 145, Arg 127, and the carbonyl oxygen of residue 197 are consistent with recent crystallographic studies of Lipscomb and co-workers.[56,57] The presumed stabilizing interactions shown for the nitrogen of the scissile bond, however, are based on presumed analogies with thermolysin and, as such, differ from the mechanistic scheme proposed recently by Christianson, David, and Lipscomb.[56]

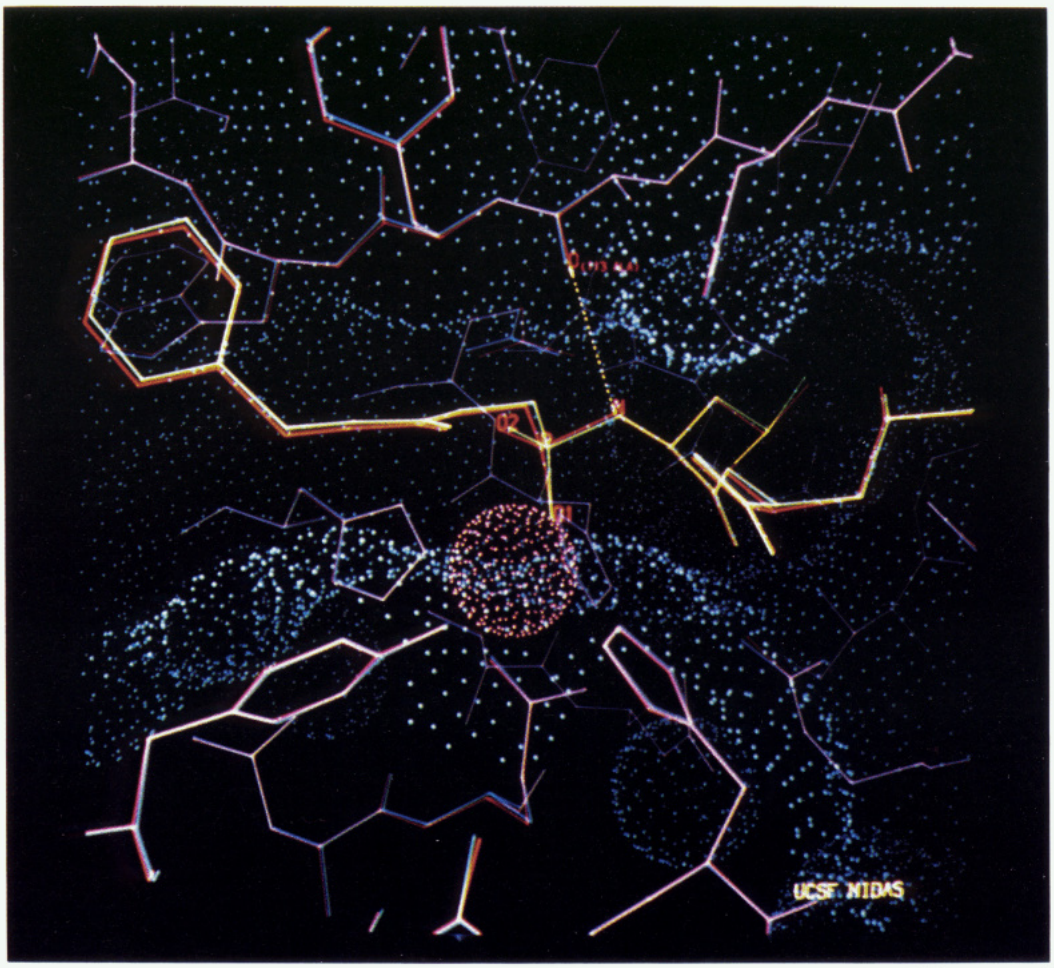

Figure 7. Superposition of two thermolysin–inhibitor complexes that differ by a single hydrogen bond (drawn as a broken yellow line). The complex of thermolysin with ZG^{P}LL is drawn red. In the complex with ZG^{P}(O)LL, the enzyme is drawn blue and the inhibitor green. The surface of the enzyme is drawn stippled (blue) as is the zinc ion (mauve).[27] (Figure courtesy of Drs. P. A. Bartlett and P. A. Bash.)

correspond exactly, either chemically or spatially.

In view of the similarity between the central catalytic steps in the presumed mechanisms of thermolysin and carboxypeptidase A, it is of interest to look for parallels in the secondary aspects of catalysis, in particular in substrate binding and stabilization of the transition state. His 231 of thermolysin and Arg 127 of carboxypeptidase A appear to play similar roles. Both are thought to hydrogen bond the incoming substrate and to stabilize the hydrated peptide in the transition state (Figures 3a–c and 6a–c).[41,57] In the vicinity of the scissile nitrogen, however, the parallels between the two enzymes are not so apparent. In the case of thermolysin, the peptide oxygen of Ala 113 and the side-chain

(57) Christianson, D. W.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 6840. Christianson, D. W.; Lipscomb, W. N. *J. Am. Chem. Soc.* **1986**, *108*, 4998.

oxygen of Asn 112 are both well positioned to accept hydrogen bonds from the doubly protonated tetrahedral nitrogen in the transition state. Glu 143 might also help stabilize this intermediate. There are precedents for these hydrogen bonds in observed thermolysin–inhibitor complexes[16,19,22,25,33] (Figures 2 and 5). For carboxypeptidase A, however, the interactions are more speculative. It is suggested in Figure 6c that the doubly protonated (cationic) nitrogen in the transition state could be stabilized by interactions with Glu 270 and Tyr 248. [This differs from the recent report of Christianson et al.[56] in which no stabilizing interactions of the cationic nitrogen are shown.]

Energetics of Enzyme–Inhibitor Interactions

By comparing the binding of appropriately matched pairs of thermolysin inhibitors, it has been possible to examine the importance of one specific hydrogen bond between enzyme and inhibitor. Bartlett and Marlowe[26] compared the inhibition constants of a series of phosphorus-containing analogues of the peptides carbobenzoxy-Gly-Leu-X in which the Gly-Leu peptide bond was replaced either with a phosphonate ester ($-PO_2^-$–O–) or a phosphonamide ($-PO_2^-$–NH–) linkage. In the five pairs that were analyzed, the peptide analogue bound approximately 1000-fold more tightly than its ester counterpart.

One pair of these inhibitors, Cbz-Gly^P-O-Leu-Leu (ZG^P(O)LL; K_i = 9000 nM) and Cbz-Gly^P-NH-Leu-Leu (ZG^{P}LL; K_i = 9.1 nM) was examined crystallographically, and the two counterparts were shown to bind in a virtually superimposable manner (Figure 7).[27] The two inhibitors differ in that the peptide analogue, ZG^{P}LL, has a hydrogen bond (3.0 Å) to the backbone carbonyl oxygen of Ala 113 whereas the ester counterpart, ZG^P(O)LL, lacks this interaction. The intrinsic binding energy of this specific hydrogen bond can therefore be assigned as 4.1 kcal/mol. Theoretical estimates of the strength of this interaction[58] agree with the experimental value. It may be that the strength of this hydrogen bond is maximized because the other interactions between inhibitor and enzyme are sufficient to bring the interacting moieties into alignment even in the absence of the hydrogen bond. This is an example of the principle of "local concentration".[59] At face value the result suggests that strong hydrogen bonds do not necessarily require the participation of charged groups, as suggested by Fersht and co-workers,[60] but other effects also need to be considered.[61]

Kinetics of Inhibitor Binding

An interesting aspect of the binding of phosphorus-containing inhibitors to thermolysin is that some of these compounds not only bind very tightly but they also bind very slowly. Cbz-Phe^P-Leu-Ala (ZF^{P}LA; Figure 2f), for example, has an affinity constant K_i = 0.068 nM and a second-order rate constant k_{on} = 1000 M^{-1} s^{-1}.[24] Bartlett and Marlowe[24] have shown that the replacement of the phenylalanine in the S_1 position with a glycine results in a more normal rate of binding. For example, ZG^{P}LA has an affinity constant K_i = 16.5 nM and a second-order rate constant in excess of 10^5 M^{-1} s^{-1}. Other inhibitors of this series with an L-amino acid at the S_1 position are slow binders.

Determination of the respective structures of the slow-binding inhibitor, ZF^{P}LA, and the normal-binding counterpart, ZG^{P}LL, showed that these two inhibitors bind to thermolysin with different configurations in the S_1–S_2 subsites (Figures 2 and 5).[25] For ZF^{P}LA the carbonyl oxygen of the carbobenzoxy group is hydrogen bonded directly to the backbone amide of Trp 115 (Figures 2f and 5b). In ZG^{P}LL, on the other hand, the carbonyl group is rotated 117° and a water molecule is interposed between the inhibitor and the enzyme (Figures 2g and 5c).

The three-dimensional structure of thermolysin is practically unchanged on binding either the slow-binding or the faster-binding inhibitor. This precludes a slow isomerization of the enzyme as the basis for slow binding. The analysis of the slowly bound inhibitor also shows that it does not have an unusually strained or rare configuration when bound to thermolysin. This tends to rule out models for slow binding in which the inhibitor cannot bind until it assumes some energetically unfavorable or improbable configuration. The conformational analysis of the slow-binding inhibitor does, however, show that rotation of the carbamate group is restricted to a "window" of about 90°. In contrast, the carbonyl of ZG^{P}LL can sample a range of about 200° and can adopt a conformation with the water molecule interposed between it and the enzyme. This conformation is not possible for ZF^{P}LL, which must therefore displace the water molecule in order to penetrate fully the active-site cleft. It is presumed that the bulky phosphonamide group, together with the hindered carbamate group, tend to prevent facile extrusion of the water molecule during the binding of the inhibitor. Rather, the water must be already displaced from its "rest" position (a rare event) in order for the inhibitor to penetrate the active-site cleft.[25] Rich[62] has proposed that a slowly displaced solvent molecule is responsible for the slow binding of pepstatin to pepsin. It should be noted that the above argument for slow binding does not apply to a substrate. A substrate has a trigonal carbonyl group instead of the tetrahedral phosphonamidate linkage. The carbonyl group is not only less bulky than the phosphonamide group, it also allows greater freedom of rotation.

Rational Drug Design

An appreciation of the structure and function of the zinc peptidases has been instrumental in leading to the development of captopril[63,64] and enalapril,[65] two widely used drugs for the control of hypertension.

To explore the potential of rational drug design in a case in which the target enzyme was known, Hangauer

(58) Bash, P. A.; Singh, U. C.; Brown, F. K.; Langridge, R.; Kollman, P. A. *Science (Washington, D.C.)* **1987**, *235*, 574.
(59) Creighton, T. E. *Biopolymers* **1983**, *22*, 49.
(60) Fersht, A. R.; Shi, J.-P.; Knill-Jones, J.; Lowe, D. M.; Wilkinson, A. J.; Blow, D. M.; Brick, P.; Carter, P.; Waye, M. M. Y.; Winter, G. *Nature (London)* **1985**, *314*, 235.
(61) Fersht, A. R. *Trends Biol. Sci.* **1987**, *12*, 301.
(62) Rich, D. H. *J. Med. Chem.* **1985**, *28*, 263.
(63) Ondetti, M. A.; Rubin, B.; Cushman, D. W. *Science (Washington, D.C.)* **1977**, *196*, 441. Cushman, D. W.; Cheung, H. S.; Sabo, E. F.; Ondetti, M. A. *Biochemistry* **1977**, *16*, 5484.
(64) Petrillo, E. W., Jr.; Ondetti, M. A. *Med. Res. Rev.* **1982**, *2*, 1.
(65) Patchett, A. A.; Harris, E.; Tristham, E. W.; Wyvratt, M. J.; Wu, M. T.; Taub, D.; Peterson, E. R.; Ikeler, T. J.; ten Broeke, J.; Payne, L. G.; Ondeyka, D. L.; Thorsett, E. D.; Greenlee, W. J.; Lohr, N. S.; Hoffsommer, R. D.; Joshua, H.; Ruyle, W. V.; Rothrock, J. W.; Aster, S. D.; Maycock, A. L.; Robinson, F. M.; Hirschmann, R. *Nature (London)* **1980**, *288*, 280. Patchett, A. A.; Cordes, E. H. *Adv. Enzymol.* **1985**, *57*, 1.

et al.[34] used computer graphics to design new inhibitors based on the known complex of CLT (Figure 2k) with thermolysin. One such compound (CCPALT; Figure 2l) was synthesized and found to bind to thermolysin 5-fold more tightly than its parent.[34] This is a particularly encouraging result. Unfortunately, it was not possible to confirm the mode of binding experimentally because the compound was too large to be accommodated within the crystals of thermolysin or possibly because it was slowly hydrolyzed. The species that was observed to bind to crystalline thermolysin was that shown outside the parentheses of Figure 2l and was present in small amounts as a synthesis intermediate.[34]

Concluding Remarks

High-resolution X-ray crystallography has been used to determine the modes of binding to thermolysin of a series of different inhibitors, including dipeptides, mercaptans, hydroxamates, *N*-carboxymethyl peptides, and phosphonamidates. The interactions displayed by such inhibitors illustrate interactions that are presumed to occur between the enzyme and its substrates during catalysis. The crystallographic analyses, together with model building, suggest a detailed stereochemical mechanism of action for thermolysin and, by analogy, other zinc proteases such as carboxypeptidase A and the angiotensin-converting enzyme. Recent analysis of a series of phosphonamidates, which are presumed to be transition-state analogues, has shown that chemically similar inhibitors can adopt dissimilar modes of binding. These different configurations provide a rationalization for large differences in the kinetics of binding that are observed for these inhibitors. Comparison of matched pairs of inhibitors allows the importance of a single hydrogen bond to be determined. Experiments with thermolysin as a test case suggest that knowledge of the three-dimensional structure of an enzyme or receptor will greatly facilitate the rational design of drugs directed at such targets.

The structural studies of thermolysin reviewed here represent the work of a number of talented students and research associates, including M. C. Bolognesi, P. M. Colman, H. M. Holden, M. A. Holmes, J. N. Jansonius, W. R. Kester, A. F. Monzingo, S. Roderick, D. E. Tronrud, and L. H. Weaver. I have also benefited from advice and material help from many colleagues, including Drs. P. A. Bartlett, D. G. Hangauer, H. Neurath, M. A. Ondetti, A. A. Patchett, J. C. Powers, B. P. Schoenborn, B. L. Vallee, and R. Wolfenden. I am also most grateful to Drs. P. A. Bartlett, P. A. Bash, H. M. Holden, S. J. Remington, and D. E. Tronrud for providing the color figures. The work was supported in part by grants from the National Institutes of Health and the National Science Foundation.