Structural Analysis of the Inhibition of Thermolysin by an Active-Site-Directed Irreversible Inhibitor†

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ABSTRACT: The mode of binding of the irreversible thermolysin inhibitor $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 [Rasnick, D., & Powers, J. C. (1978) *Biochemistry 17*, 4363–4369] has been determined by X-ray crystallography at a resolution of 2.3 Å and the structure of the covalent complex refined to give a crystallographic residual of 17.0%. This is the first such structural study of an active-site-directed covalent complex of a zinc protease. As anticipated by Rasnick and Powers, the inhibitor alkylates Glu-143 in the thermolysin active site, and the hydroxamic acid moiety coordinates the zinc ion. The formation of the covalent complex is associated with a significant shift in a segment of the polypeptide backbone in the vicinity of the active site. This conformational adjustment appears to be necessary to relieve steric hindrance which would otherwise prevent alkylation of Glu-143. It is suggested that this steric hindrance, which occurs for thermolysin but would not be expected for carboxypeptidase A, accounts for the previously inexplicable difference in reactivity of these two metalloproteases toward *N*-haloacetyl amino acids. The relevance of this steric hindrance to the mechanism of catalysis is discussed. In agreement with previous results [Kester, W. R., & Matthews, B. W. (1977) *Biochemistry 16*, 2506–2516], it appears that steric hindrance prevents the direct attack of Glu-143 on the carbonyl carbon of an extended substrate, therefore ruling out the anhydride pathway in thermolysin-catalyzed hydrolysis of polypeptide substrates and their ester analogues.

Zinc metalloproteases occur widely in nature and are involved in many important physiological processes. The role of carboxypeptidases A and B in digestion has long been recognized. Collagenase is thought to be the destructive agent in arthritis (Harris & Krone, 1974), and another zinc protease, the angiotensin-converting enzyme, is important in regulating blood pressure (Peach, 1977). It has been shown that the active site of thermolysin has striking similarities to that of carboxypeptidase A (Kester & Matthews, 1977a), even though the backbone conformations of the two enzymes are otherwise very different. Also, known inhibitors of one member of this class of enzymes, when modified according to differences in enzyme specificity, are often found to be good inhibitors of other members of the class (Kam et al., 1979; Nishino & Powers, 1979; Holmquist & Vallee, 1979; Maycock et al., 1981). Thus, all these zinc proteases may have similarities in their active sites and in their mechanisms of action. Recent discussions of the structure and mechanism of catalysis of the zinc proteases have been given by Lipscomb (1980), Rees et al. (1981), Rees & Lipscomb (1981), Holmes & Matthews (1981), and Kuo & Makinen (1982).

We here describe the mode of binding to thermolysin of the covalent inhibitor $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 (Rasnick & Powers, 1978). The inhibitor is patterned after the *N*-haloacetyl amino acid inhibitors of carboxypeptidase A developed by Hass & Neurath (1971a,b) but modified by the inclusion of an *N*-hydroxy function to have enhanced zinc-binding capability. This is the first crystallographic analysis of the irreversible inhibition of any of the zinc proteases and, as such, provides a general model for the mode of action of this type of inhibitor.

Materials and Methods

Thermolysin, 3 times recrystallized, was obtained from Calbiochem. The inhibitor was a gift of Dr. J. C. Powers (Rasnick & Powers, 1978).

Native crystals of thermolysin were obtained as described previously (Colman et al., 1972) and were stored in a mother liquor of 0.01 M calcium acetate, 0.01 M tris(hydroxymethyl)aminomethane–acetate (Tris–acetate), and 5 or 7% v/v dimethyl sulfoxide, pH 7.2. Crystals of the thermolysin–inhibitor complex were obtained by soaking native crystals in mother liquor in which the inhibitor had been dissolved at a concentration of 50 μM. The crystals were soaked in the inhibitor solution for 1–2 days and then transferred to the standard mother liquor before use.

The diffraction data for the inhibitor complex were collected by using the method of precession photography. The X-ray source was an Elliot GX-6 rotating anode generator, and crystals were exposed for 48 h. A summary of the data collection statistics for the complex is given in Table I.

The usual difference electron density map with coefficients $F_{complex} - F_{native}$ and phase angles from isomorphous replacement was calculated but was not suitable for model building since the native enzyme has many water molecules bound in the active site which are displaced by bound inhibitors (Kester & Matthews, 1977b). Instead, a map with coefficients $2F_{complex} - F_{native}$ was calculated, and a Kendrew–Watson model was fitted to the map by using an optical comparator (Richards, 1968; Colman et al., 1972). The D stereoisomer of the inhibitor was used, as it gave a better fit to the map. Preliminary inhibitor coordinates were obtained by placing markers on the map in the corresponding density, guided by the wire model.

Refinement of the thermolysin–inhibitor complex was based on the method of restrained least squares by using a set of programs written and developed in this laboratory by Dr. L. F. Ten Eyck and D. E. Tronrud (unpublished results). Starting coordinates for the protein were taken from the refinement of native thermolysin at 1.6 Å resolution (Holmes & Matthews, 1982). "Ideal" stereochemistry for the protein and the inhibitor was based on the values collected by L. F. Ten Eyck (unpublished results) and Bowen et al. (1958). Results of the refinement are summarized in Table II.

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Table I: Intensity Statistics for Inhibitor Data Set

no. of film packs	18
no. of reflections	12268
resolution (Å)	2.3
av R_{sym}[a] (%)	7.1
R_{merge}[b] (%)	3.5
av isomorphous difference (%)	9.6

[a] $R_{sym} = \Sigma|I-\bar{I}|/\Sigma I$ for symmetry-related intensities recorded on the same film. [b] $R_{merge} = \Sigma|F-\bar{F}|/\Sigma F$ for reflections measured on different films.

Table II: Refinement Statistics for the Inhibitor Complex

initial R value[a] (%)	20.5
final R value (%)	17.0
rms deviation from ideality	
bond length (Å)	0.021
bond angle (deg)	2.9
planarity (trigonal) (Å)	0.022
planarity (other planes) (Å)	0.024
torsion angle (deg)	27.0
no. of refinement cycles	
coordinate refinement	16
temp factor refinement	4
no. of reflections used	
10.0–2.3 Å	12094

[a] R value $= \Sigma|F_{obsd}-F_{calcd}|/\Sigma|F_{obsd}$.

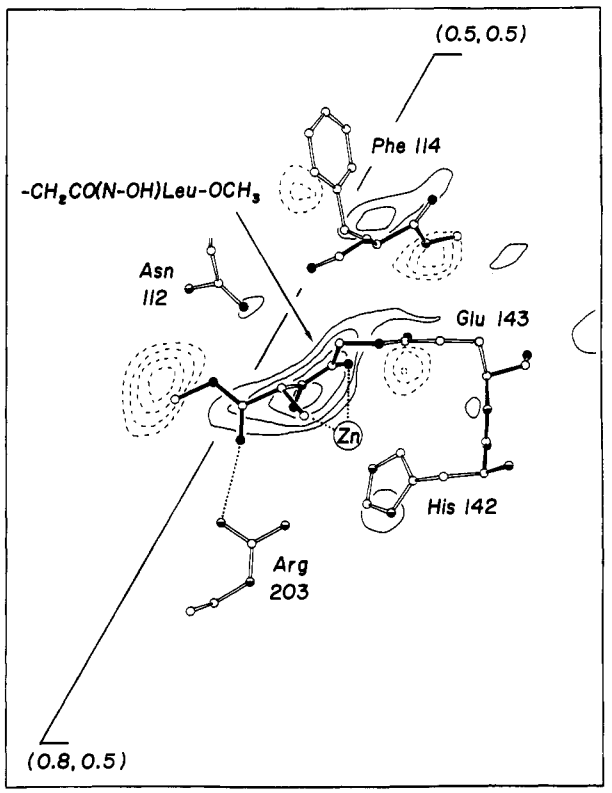

FIGURE 1: Section $z = -0.039$ of the difference electron density map passing close to the zinc ion. Positive contours, drawn solid, are at levels of 2σ, 3σ, 4σ, etc., and negative contours, drawn broken, are at levels of -2σ, -3σ, -4σ, etc., where σ is the root mean square value of the difference density throughout the unit cell. The obvious negative feature at the left of the inhibitor is due to the displacement of solvent. The inhibitor and the protein backbone are drawn in solid bonds; amino acid side chains are drawn with open bonds. Carbon atoms are drawn open, nitrogen half solid, and oxygen solid.

Results

Binding of the Inhibitor. The difference map with coefficients $F_{complex} - F_{native}$ clearly showed that the inhibitor was bound in the active-site region of the enzyme. The difference density in this region exceeded 6σ where σ is the root mean square density throughout the unit cell. Also, the highest positive and negative features exceeded, by a factor of 2, any other features away from the active site. Part of the inhibitor was seen to be interacting with the zinc ion, and it was also apparent that the inhibitor was covalently bonded to the side chain of Glu-143 (Figure 1). There was positive and negative density corresponding to a shift of residues 114–116 away from the inhibitor.

Model fitting to the $2F_{complex} - F_{native}$ difference map showed that the hydroxamic acid group of the inhibitor was coordinated to the zinc, possibly as a bidentate ligand, as had been seen with other hydroxamic acid inhibitors (Holmes & Matthews, 1981). The leucine side chain occupied the hydrophobic pocket that gives thermolysin its primary specificity. Each of these features is consistent with the mode of inhibition postulated by Rasnick & Powers (1978).

Refinement of the Inhibited Complex. The structure of the inhibited complex was refined to a crystallographic R value of 17.0% (Table II). The root mean square shift for the protein during refinement of the complex was 0.10 Å. Table III lists

Table III: Refined Inhibitor Coordinates[a]

atom	X	Y	Z	B
CA1	51.24	20.09	−3.92	24.69
C1	51.88	19.81	−5.24	31.50
O1	51.70	20.42	−6.36	32.32
N2	52.58	18.77	−5.14	34.53
OH2	53.23	18.38	−6.25	24.50
CA2	52.76	18.05	−3.81	24.54
CB2	53.81	18.72	−2.96	27.53
CG2	53.70	18.37	−1.48	21.16
CD12	54.81	19.20	−0.91	20.61
CD22	52.33	18.60	−0.76	21.21
C2	53.28	16.61	−3.96	21.82
O2	54.49	16.52	−3.70	14.50
O3	52.41	15.58	−4.45	34.01
C3	53.01	14.23	−4.47	23.09
H_2O 362	50.01	21.26	−8.05	27.19
H_2O 394	50.38	17.80	−7.43	49.13

[a] The coordinates are in angstroms relative to a right-handed coordinate system, with the origin at the crystallographic origin and X parallel to a^*, Y parallel to b, and Z parallel to c. The numbering scheme is as follows:

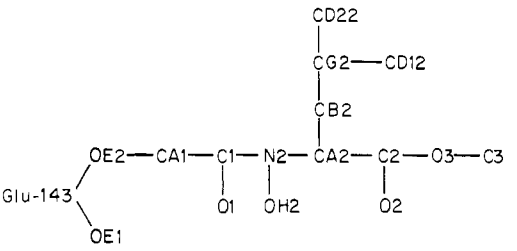

Table IV: Thermolysin–Inhibitor Distances

protein	inhibitor	distance (Å)
Asn-112 OD1	O3	3.2
Ala-113 O	CA1	3.1
Glu-143 OE1	O1	2.4 (H)[a]
His-146 NE2	O1	3.4
Glu-166 OE1	OH2	3.3
Arg-203 NH1	O2	3.1 (H)
His-231 NE2	OH2	2.8 (H)
Zn^{2+}	O1	2.8
Zn^{2+}	OH2	2.5
H_2O 362	O1	2.5 (H)
H_2O 362	Glu-143 OE1	3.1 (H)

[a] The letter H denotes a presumed hydrogen bond.

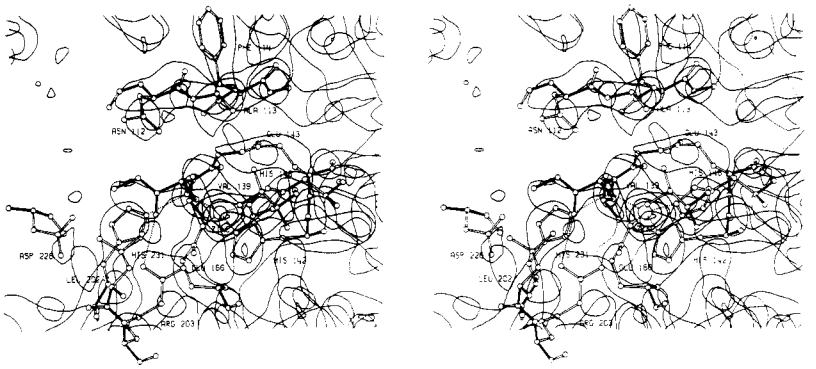

FIGURE 2: Stereo drawing of the $2F_{complex} - F_{native}$ density superimposed on the bound inhibitor, drawn at an early stage of refinement. Contours are drawn at levels of 2σ, 4σ, and 6σ.

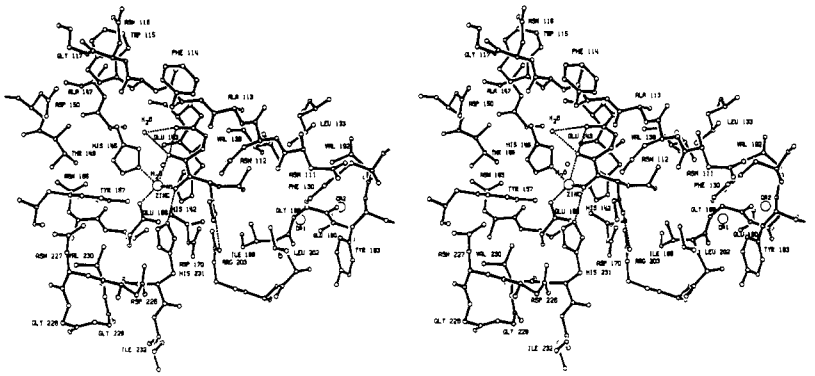

FIGURE 3: Extended stereo view of the thermolysin active site with the inhibitor bound to Glu-143. The direction of view is from the left of that shown in Figures 1 and 2.

the refined inhibitor coordinates. The complete list of refined coordinates has been deposited at the Protein Data Bank (Bernstein et al., 1977). The estimated accuracy of the complex coordinates is about 0.2 Å. Table IV gives selected distances between protein atoms and inhibitor atoms in the refined complex. Figure 2 is a stereo drawing of the inhibitor in the active site superimposed on electron density from the $2F_{complex} - F_{native}$ map, and Figure 3 is a more extended view of the structure of the complex in the vicinity of the active site.

The coordination at the zinc by the hydroxamate function is seen to be quite distorted. The *N*-hydroxyl oxygen is 2.5 Å from the zinc and is hydrogen bonded by the imidazole of His-231. The carbonyl oxygen is at a distance of 2.8 Å from the zinc and could be considered as an additional weak ligand. There is a water molecule which forms a hydrogen-bonding bridge between the Glu-143 carboxyl group and the inhibitor carbonyl oxygen (Figure 3).

Discussion

In general terms, the interaction between the inhibitor and the enzyme resembles that seen previously for noncovalent thermolysin–inhibitor complexes [e.g., see Kester & Matthews (1977b) and Holmes & Matthews (1981)] and confirms the mode of inhibition proposed by Rasnick & Powers (1978). In particular, the inhibitor is seen to alkylate Glu-143, and the hydroxamic acid functional group is seen to bind the active-site zinc. Apart from the formation of the covalent linkage, the enzyme–inhibitor complex differs in two respects from the noncovalent complexes studied previously. In the first place, the interaction of the hydroxamic acid group with the zinc is quite distorted, relative to that seen with, for example, L-Leu-NHOH (Holmes & Matthews, 1981). For the reversible hydroxamate inhibitors, the *N*-hydroxyl function and the carbonyl function are both within 2.0–2.3 Å of the zinc, to give overall pentacoordinate ligation. In contrast, the covalent complex has respective oxygen–metal distances of 2.5 and 2.8 Å. It would seem likely that in the initial complex of the inhibitor with the enzyme, a well-defined pentacoordinate complex is formed, and the subsequent alkylation results in the observed distortion.

The second difference between the covalent and noncovalent inhibitor complexes with thermolysin concerns the nature of the conformational changes in the protein. In the thermolysin–inhibitor complex studied heretofore, including dipeptides, phosphoramides, hydroxamates, mercaptans, and succinates, there has been minimal steric hindrance between the inhibitor and the protein, and the apparent structural adjustments have been minor [see, for example, Holmes & Matthews (1981), Monzingo & Matthews (1982), and Bolognesi & Matthews (1979)]. In contrast, the formation of the covalent complex results in significant adjustment in the vicinity of the active site which seems to be directly related to steric hindrance between the inhibitor and the protein. To illustrate the location and magnitude of these movements, we show, in Figure 4, the difference between the refined protein coordinates of the native thermolysin structure and those for the refined thermolysin–inhibitor complex. The difference gives the change in the position of each atom in the protein on binding the inhibitor. Two regions of the protein move significantly. One of these is the side chain of Glu-143, i.e., the site of alkylation. The second region is more extensive, extending from Ala-113 through Asn-116. This segment of the protein is in a fully extended conformation and is located "above" the active-site zinc ion (Figures 2 and 3). The segment forms the final strand in an extended β sheet and appears to contribute to the interaction of the protein with extended substrates [Kester & Matthews, 1977b; see also Figure 8 of Colman et al. (1972)]. On formation of the covalent complex, this segment of the

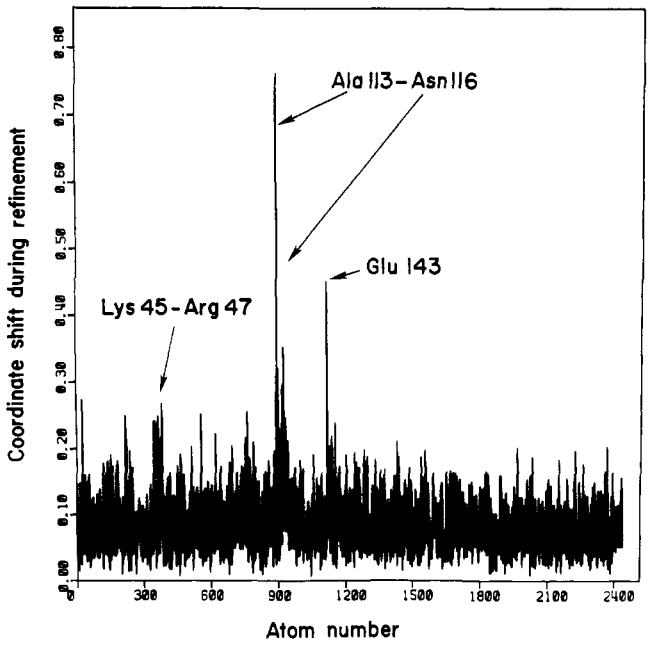

FIGURE 4: Plot showing the difference in position of all atoms in the thermolysin–inhibitor complex relative to their position in native thermolysin, as estimated by the shift of each atom during the refinement of the complex. The "background" level of 0.1–0.2 Å should not be taken to imply real displacements. Rather, the background level provides a measure of the difference in coordinates which can be attributed to the independent refinements of the native and complex structures. The approximate residue number can be obtained by dividing the atom number by 7.8.

protein moves about 0.2–0.3 Å away from the bound inhibitor (i.e., "upward" in Figures 2 and 3). Also, the peptide group between Ala-113 and Phe-114 rotates by about 15°, resulting in a net movement of the carbonyl oxygen of about 0.76 Å (Figure 4). This shift appears to be induced by straightforward steric effects. If Ala-113 were to remain as placed in native thermolysin, its peptide carbonyl oxygen would be 2.3 Å from the methylene carbon CA1. As a result of the structural adjustment, this distance increases to the acceptable value of 3.1 Å.

The fact that the alkylation of Glu-143 by a substrate analogue is possible, at least under certain circumstances, is of interest from a mechanistic point of view, since it might provide a *structural* (as opposed to chemical) precedent for the existence of an acyl-enzyme intermediate during catalysis. There have been two principal schools of thought concerning the modes of action of carboxypeptidase A and thermolysin. One possibility, the "anhydride" pathway, presumes the direct attack of a glutamate moiety (Glu-143 in the case of thermolysin; Glu-270 for carboxypeptidase A) on the carbonyl carbon of the scissile bond, to form a covalent acyl-enzyme intermediate [e.g., see Quiocho & Lipscomb (1971), Makinen et al. (1979, 1982), Kuo & Makinen (1982), and Rees et al. (1981)]. In the alternative "general base" scheme, the glutamate is presumed to promote the attack of a water molecule on the carbonyl carbon of the substrate, forming a tetrahedral noncovalently linked enzyme–substrate complex [e.g., see Quiocho & Lipscomb (1971), Breslow & Wernick (1977), and Kester & Matthews (1977b)] [see also Holmes & Matthews (1981) and Makinen et al. (1982) for discussions of the possible roles of pentacoordinate zinc intermediates in catalysis].

In the case of carboxypeptidase A, crystallographic studies have not distinguished between the respective catalytic schemes (Lipscomb, 1980), but for thermolysin such studies provided two lines of evidence in support of a noncovalent pathway for peptide hydrolysis. First, Glu-143 was seen to be located within a rather narrow cleft in the enzyme surface such that the approach of the scissile bond of the substrate sufficiently close to allow the formation of a covalent adduct was sterically impossible without rather large conformational changes in the protein (Kester & Matthews., 1977b). [In the case of carboxypeptidase A, the active site is more "open" than is the case in thermolysin, so that the same structural restrictions do not apply [e.g., see Figures 1–3 of Kester & Matthews (1977a)].] Second, the geometry of binding of the tetrahedral phosphoramide moiety of the naturally occurring thermolysin inhibitor phosphoramidon provided a structural analogue of the tetrahedral intermediate which would be formed following attack of a water molecule or hydroxide ion on the carbonyl carbon (Weaver et al., 1977).

Now, in what might seem to be a refutation of the above argument, we see that it is possible to form a covalent adduct between Glu-143 of thermolysin and an inhibitor which is, in several respects, an analogue of a substrate. The first point to be noted, however, is that the binding of the inhibitor is associated with obvious conformational changes in the protein (Figures 2 and 4). These changes consist of a bodily shift of 0.2–0.3 Å in an extended polypeptide segment and a 15° rotation of a peptide group, which is, apparently, necessary to relieve prohibitively short contacts between enzyme and inhibitor. In the case of an acyl-enzyme intermediate for a polypeptide substrate, the steric interference would be much worse than is the case with the inhibitor described here. In particular, the CA1 methylene carbon of the inhibitor that is the source of the steric hindrance described above would be replaced by a tetrahedral oxyanion group which is much more bulky. Furthermore, the presence of the adjacent α carbon and its R group would also appear to cause severe steric interference between enzyme and substrate that is avoided by the design of the present inhibitor. Therefore, it seems certain that the formation of an acyl-enzyme intermediate for a polypeptide substrate would necessitate much larger conformational changes of the protein than are seen here.

However, it could be argued that such conformational changes are permitted, that the energy cost is negligible, and that these steric arguments do not rule out the anhydride pathway for thermolysin. An observation which may be relevant in this connection concerns the different activity of alkylating agents toward carboxypeptidase A and thermolysin. The compound *N*-(bromoacetyl)-L-*N*-methylphenylalanine has been shown to modify Glu-270 of carboxypeptidase A (Hass & Neurath, 1971a,b), but analogous compounds for thermolysin, including $BrCH_2CO$-Phe-OCH_3, $BrCH_2CO$-L-MeLeu-OCH_3, and $BrCH_2CO$-L-MeLeu-L-Ala-OCH_3, do not react with the enzyme (Rasnick & Powers, 1978). This unexpected difference in reactivity toward the respective enzymes can now be rationalized in terms of the differences in accessibility of Glu-143 and Glu-270 in the respective enzymes to the alkylating group. In carboxypeptidase A, the active site is relatively "open" and would permit free access of the inhibitor to Glu-270, whereas in thermolysin, access of the inhibitor to Glu-143 is more difficult and, as judged by the present study, necessitates a significant structural adjustment of the protein. Since alkylation of thermolysin by the above compounds does not occur, it can be inferred that the energy of (noncovalent) binding of these compounds is insufficient to induce the required structural adjustment of the protein (or, in other words, the ground state of the protein does not favor the initial binding of these compounds). However, in the case of the compound

containing the hydroxamic acid function, the enhanced affinity for the enzyme is, apparently, sufficient to induce the requisite structural change in the protein. Since the incorporation of the hydroxamic group in a typical peptide inhibitor of thermolysin enhances the inhibition constant by about 3 orders of magnitude (Nishino & Powers, 1978, 1979), the binding energy required to induce the conformational change in the protein can be *very roughly* estimated as about 4 kcal. We have pointed out that in order for a peptide substrate to form an acyl-enzyme intermediate a much larger conformational adjustment in the protein would be required, and we speculate that this might require even more binding energy, although there is no experimental evidence to support this idea.

In summary, the binding of this covalent inhibitor to thermolysin confirms the mode of binding inferred by Rasnick & Powers (1978) and shows that covalent modification of Glu-143 by a substrate analogue is associated with a significant conformational adjustment of the protein. Much larger structural changes would be necessary in order to form an acyl-enzyme complex with a true substrate. We suggest that such structural changes are unlikely and that the anhydride mechanism of catalysis for the thermolysin-catalyzed hydrolysis of polypeptides and their ester analogues is improbable.

Acknowledgments

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