# Binding of *N*-Carboxymethyl Dipeptide Inhibitors to Thermolysin Determined by X-ray Crystallography: A Novel Class of Transition-State Analogues for Zinc Peptidases†

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ABSTRACT: The mode of binding of the specific thermolysin inhibitor *N*-(1-carboxy-3-phenylpropyl)-L-leucyl-L-tryptophan ($K_I \sim 5 \times 10^{-8}$ M) [Maycock, A. L., DeSousa, D. M., Payne, L. G., ten Broeke, J., Wu, M. T., & Patchett, A. A. (1981) *Biochem. Biophys. Res. Commun. 102*, 963–969] has been determined by X-ray crystallography and refined to an *R* value of 17.1% at 1.9-Å resolution. The inhibitor binds to thermolysin with both oxygens of the *N*-carboxymethyl group liganded to the zinc to give overall pentacoordination of the metal. The bidentate ligation of the inhibitor differs from the monodentate binding seen previously for carboxylate–zinc interactions in thermolysin and is closer to the bidentate geometry observed for the binding of hydroxamates [Holmes, M. A., & Matthews, B. W. (1981) *Biochemistry 20*, 6912–6920]. The geometry of the inhibitor and its interactions with the protein have a number of elements in common with the presumed transition state formed during peptide hydrolysis. The observed zinc ligation supports the previous suggestion that a pentacoordinate intermediate participates in the mechanism of catalysis. However, the α-amino nitrogen of the inhibitor is close to Glu-143, suggesting that this residue might accept a proton from an attacking water molecule (as proposed before) and subsequently donate this proton to the leaving nitrogen. By analogy with thermolysin, it is proposed that a related mechanism should be considered for peptide cleavage by carboxypeptidase A. In such a mechanism, Glu-270 of carboxypeptidase A would promote the attack of a water molecule on the carbonyl carbon of the substrate, possibly in concert with the zinc via a pentacoordinate complex. Glu-270 would then act as a proton shuttle to transfer the proton to the leaving nitrogen. Tyr-248 of carboxypeptidase A would not act as a proton donor, as has generally been supposed [Lipscomb, W. N. (1982) *Acc. Chem. Res. 15*, 232–238] but could participate in other ways including substrate binding and alignment and perhaps in stabilization of the transition state.

Recently, Patchett et al. (1980) have introduced *N*-carboxymethyl dipeptides as a new class of potent and specific inhibitors of the angiotensin converting enzyme. The efficacy of the inhibitors was interpreted to result from their behavior as transition-state analogues.

Acting on the evidence that a number of the zinc-requiring peptidases, including carboxypeptidase A, thermolysin, and the angiotensin converting enzyme, have similarities in their modes of inhibition and, presumably, in their catalytic mechanisms (Cushman et al., 1977; Ondetti et al., 1977, 1979; Kester & Matthews, 1977a; Kam et al., 1979; Holmquist & Vallee, 1979), Patchett and co-workers adapted their design principle to thermolysin and showed that *N*-(1-carboxy-3-phenylpropyl)-L-leucyl-L-tryptophan (Figure 1) (hereafter CLT)[1] is indeed an excellent inhibitor of this metalloendopeptidase ($K_I \sim 5 \times 10^{-8}$ M) (Maycock et al., 1981). The inhibitor combines the dipeptide Leu-Trp, as in the natural thermolysin inhibitor phosphoramidon (Suda et al., 1973), with the *N*-carboxymethyl function as shown to be conducive to tight binding to the angiotensin converting enzyme.

Part of the rationale for adapting this class of inhibitors to thermolysin was the feasibility of a crystallographic determination of the mode of inhibitor binding. Such a structure analysis has now been completed and, as discussed in this paper, reveals novel features of enzyme–inhibitor interaction that were not anticipated.

## Materials and Methods

Thermolysin was obtained from Calbiochem and crystallized as described by Holmes & Matthews (1982). The crystals were stored in a mother liquor of 0.01 M calcium acetate, 0.01 M Tris–acetate, and 7% (v/v) dimethyl sulfoxide, pH 7.2. Complexes of thermolysin with the inhibitor CLT, a gift of Dr. A. A. Patchett (Maycock et al., 1981), were obtained by soaking native crystals in mother liquor containing the inhibitor. The amount of binding was monitored by calculating (*h*0*l*) difference Fourier projections [cf. Weaver et al. (1977)].

A single thermolysin crystal soaked for 12 days with 50 μM CLT was used for three-dimensional data collection. Data were collected to 1.7-Å resolution by use of oscillation photography (Rossmann, 1979; Schmid et al., 1981). Thermolysin crystals generally grow as hexagonal rods, enabling us to make 12 exposures at one end of the crystal and then to translate the crystal horizontally to an unexposed portion, which was used for the remaining 16 exposures. An oscillation angle of 1.2° per film pack was used, and the crystals (space group $P6_122$) were rotated about the *c* axis through a net rotation of 30°. The X-ray source was a graphite-monochromatized Elliot GX-21 rotating anode generator operated at 39 kV, 130 mA, and the exposure time was 6 h per film. Intensity statistics are summarized in Table I.

## Results

*Model Building of the Protein–Inhibitor Complex.* A

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[1] Abbreviations: CLT, *N*-(1-carboxy-3-phenylpropyl)-L-leucyl-L-tryptophan; Tris–acetate, 2-amino-2-(hydroxymethyl)-1,3-propanediol acetate; Cbz-Phe, *N*-carbobenzoxy-L-phenylalanine; L-Leu-NHOH, L-leucyl-NHOH; rms, root mean square.

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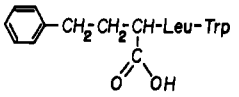

FIGURE 1: Chemical structure of CLT.

Table I: Intensity Statistics

| | |
|---|---|
| no. of film packs | 28 |
| no. of independent reflections | 23 734 |
| resolution (Å) | 1.7 |
| av $R_{sym}$[a] | 0.048 |
| av $R_{sca}$[a] | 0.039 |
| $R_{merge}$[a] | 0.069 |
| av isomorphous difference (%) | 15.2 |

[a] $R = \sum|I - \bar{I}|\sum I$. $R_{sym}$ measures the agreement between symmetry-related reflections on the same film, $R_{sca}$ measures the agreement between reflections recorded on successive films in a given film pack, and $R_{merge}$ gives the overall agreement between intensities measured on different films.

Table II: Refinement Statistics[a]

| | |
|---|---|
| resolution (Å) | 1.9 |
| initial $R$ value (20.0–1.9 Å) (%) | 22.9 |
| final $R$ value (%) | 17.1 |
| no. of refinement cycles | 13 |
| no. of reflections used | 19 842 |
| no. of atoms | 2616 |

[a] The crystallographic $R$ value is defined in the usual way and includes all observed reflections between 20.0- and 1.9-Å resolution.

difference electron density map with amplitudes $F_{PI} - F_P$ was calculated and examined for binding of the inhibitor in the active-site region. In computing this map, we used native structure amplitudes and phases, which had been calculated with active-site solvent molecules removed. Use of the calculated structure factors avoided confusion due to displacement of solvent from the active-site region [cf. Kester & Matthews (1977b)].

The electron density corresponding to the bound inhibitor was clearly seen in the difference map (Figure 2). The highest peak in the map (height $8\sigma$) lay near the location of the zinc ion and apparently represented the bound carboxylate group ($\sigma$ is the root mean square electron density throughout the unit cell). There was also a $7\sigma$ peak corresponding to the inhibitor leucyl side chain bound in the hydrophobic pocket of thermolysin. The remainder of the inhibitor density was represented by peaks of heights ranging from $4\sigma$ to $7\sigma$. The largest peak away from the active-site region was of height $4\sigma$.

An electron density map with amplitudes $2F_{PI} - F_P$ was calculated and used in model building. A brass model (Cambridge Repetition Engineers) of the inhibitor was fit to the electron density in an optical comparator (Richards, 1968; Colman et al., 1972). Markers placed in the map, with the brass model as a guide, provided approximate coordinates for the bound inhibitor.

*Refinement of the Protein–Inhibitor Complex.* The protein–inhibitor complex was refined to a crystallographic residual of 0.171 by using the energy minimization and crystallographic refinement program of Jack & Levitt (1978), modified by Dr. J. Deisenhofer (personal communication) and also in this laboratory by Dr. D. H. Ohlendorf to better enforce planarity where chemically appropriate throughout the protein.

The chirality of the substituted *N*-methyl carbon of the inhibitor used in this study may be either *R* or *S* (Maycock et al., 1981). Models with either configuration fit the electron density reasonably well. The crystallographic refinement was begun with coordinates in the *R* configuration, but during refinement, they changed to *S*, indicating that the latter configuration is most compatible with the high-resolution diffraction data.

Table III: Deviations from Ideal Geometry

| | protein | | inhibitor | |
|---|---|---|---|---|
| | no. | rms deviation | no. | rms deviation |
| bond length (Å) | 2528 | 0.021 | 37 | 0.029 |
| bond angle (deg) | 3431 | 3.6 | 47 | 2.9 |
| planarity (trigonal) (Å) | 67 | 0.063 | 1 | 0.060 |
| planarity (other planes) (Å) | 377 | 0.041 | 3 | 0.089 |

Table IV: Inhibitor Coordinates[a]

| residue | atom | $X$ | $Y$ | $Z$ | $B$ |
|---|---|---|---|---|---|
| CPP | OG2 | 52.3 | 20.3 | −6.3 | 21.9 |
| | CB2 | 52.5 | 19.1 | −6.5 | 22.6 |
| | OG1 | 53.3 | 18.7 | −7.3 | 18.3 |
| | CB1 | 50.3 | 18.1 | −6.5 | 21.0 |
| | CG | 49.8 | 16.7 | −6.7 | 28.3 |
| | CD | 48.5 | 16.4 | −7.4 | 25.7 |
| | CE1 | 48.5 | 16.4 | −8.8 | 27.1 |
| | CZ1 | 47.3 | 16.0 | −9.5 | 29.1 |
| | CH | 46.1 | 15.8 | −8.8 | 23.5 |
| | CE2 | 47.4 | 16.1 | −6.7 | 26.1 |
| | CZ2 | 46.2 | 15.8 | −7.4 | 27.8 |
| | CA | 51.6 | 18.1 | −5.7 | 24.5 |
| Leu | N | 51.5 | 18.2 | −4.2 | 21.3 |
| | CB | 52.6 | 18.1 | −2.0 | 16.5 |
| | CG | 53.9 | 18.2 | −1.3 | 15.5 |
| | CD1 | 54.6 | 19.5 | −1.7 | 16.4 |
| | CD2 | 53.7 | 18.2 | 0.2 | 20.8 |
| | CA | 52.8 | 18.1 | −3.5 | 22.5 |
| | C | 53.7 | 16.9 | −3.9 | 18.4 |
| | O | 54.9 | 16.9 | −4.0 | 17.6 |
| Trp | N | 53.0 | 15.8 | −4.0 | 18.4 |
| | CB | 54.1 | 13.7 | −3.0 | 23.5 |
| | CG | 52.9 | 13.3 | −2.2 | 19.8 |
| | CD1 | 51.8 | 13.9 | −1.8 | 24.8 |
| | NE1 | 50.9 | 13.0 | −1.3 | 25.5 |
| | CE2 | 51.4 | 11.8 | −1.3 | 25.5 |
| | CZ2 | 50.9 | 10.6 | −0.9 | 28.6 |
| | CH2 | 51.8 | 9.6 | −0.7 | 30.4 |
| | CZ3 | 53.2 | 9.7 | −1.0 | 30.8 |
| | CE3 | 53.7 | 10.9 | −1.5 | 30.1 |
| | CD2 | 52.8 | 12.0 | −1.6 | 25.0 |
| | CA | 53.5 | 14.4 | −4.3 | 23.0 |
| | C | 52.6 | 13.5 | −5.1 | 27.0 |
| | O | 51.6 | 14.0 | −5.6 | 22.7 |
| | OH | 53.1 | 12.5 | −5.7 | 24.2 |
| $H_2O$ | 464 | 49.9 | 21.0 | −8.0 | 27.7 |

[a] The coordinates are in angstroms in the standard orthogonal thermolysin coordinate system (Weaver et al., 1974). The thermal parameter $B$ is in Å$^2$.

Table V: Zinc–Ligand Distances

| ligand | distance (Å) | ligand | distance (Å) |
|---|---|---|---|
| His-142 NE2 | 1.9 | inhibitor OG1 | 2.0 |
| His-146 NE2 | 2.0 | inhibitor OG2 | 2.4 |
| Glu-166 OE1 | 2.0 | | |

Refinement statistics are summarized in Table II. The deviations from ideal geometry are listed in Table III for both the protein–inhibitor complex and the inhibitor alone. Refined coordinates of the bound inhibitor are listed in Table IV. Included in the list of coordinates are those of a water molecule that is apparently hydrogen bonded to the backbone N of Trp-115 (distance 3.1 Å) and possibly to the OE1 atom of Glu-143 (distance 3.2 Å). The estimated uncertainty of the refined coordinates is 0.2 Å. The coordinates, including bound solvent, are available from the protein data bank (Bernstein et al., 1977).

*Mode of Binding of the Inhibitor.* The mode of binding of the inhibitor to the thermolysin molecule is displayed stereo-

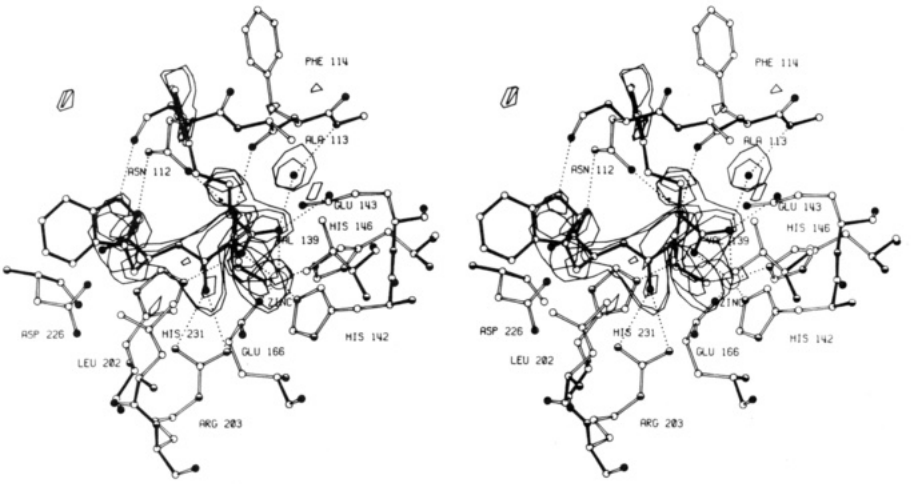


FIGURE 2: Stereo drawing of the bound inhibitor superimposed on the corresponding electron density. The electron density was calculated from a difference map with coefficients of the form $(F_{complex} - F_{nat,calcd}) \exp(i\alpha_{calcd})$, where the native amplitudes and phases were calculated from the refined native structure, with water molecules removed from the active-site region. The resolution of the map is 1.9 Å, and contours are drawn at the level of $2.5\sigma$, where $\sigma$ is the rms density throughout the unit cell. Carbon atoms are drawn open, oxygen atoms solid, and nitrogen atoms half-solid.

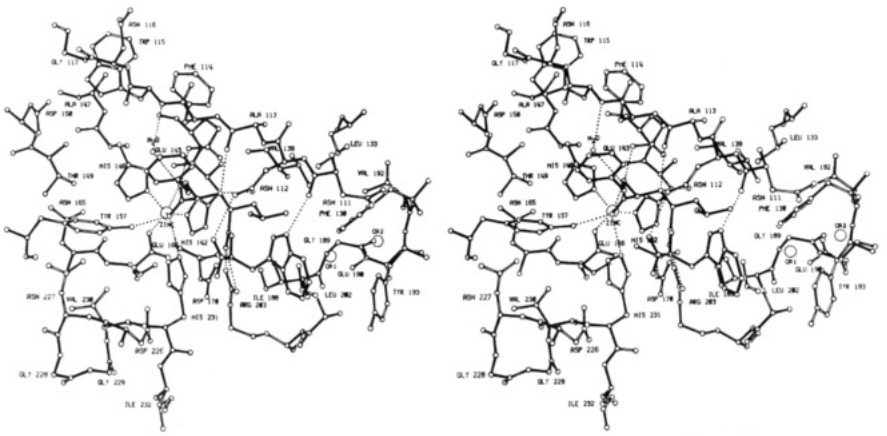

FIGURE 3: Stereo drawing of CLT bound within the extended active-site region.

Table VI: Thermolysin–Inhibitor Distances[a]

| protein | inhibitor | distance (Å) | protein | inhibitor | distance (Å) |
|---|---|---|---|---|---|
| Glu-143 OE1 | CPP OG2 | 2.8 (H) | Glu-143 OE2 | Leu N | 3.0 |
| Glu-143 OE2 | CPP OG2 | 3.0 | Asn-112 OD1 | Leu N | 3.0 (H) |
| His-146 NE2(Zn) | CPP OG2 | 3.1 | Arg-203 NEE1 | Leu O | 2.9 (H) |
| His-142 NE2(Zn) | CPP OG2 | 3.2 | Arg-203 NEE2 | Leu O | 2.9 (H) |
| Glu-166 OE1(Zn) | CPP OG1 | 2.8 | Asn-112 ND2 | Trp O | 3.1 (H) |
| Tyr-157 OH | CPP OG1 | 2.8 (H) | Asn-111 O | Trp NE1 | 2.9 (H) |
| His-231 NE2 | CPP OG1 | 2.8 (H) | Trp-115 N | $H_2O$ | 3.1 (H) |
| Ala-113 O | Leu N | 2.9 (H) | $H_2O$ | CPP OG2 | 3.1 (H) |

[a] Atoms denoted (Zn) are zinc ligands. (H) designates a presumed hydrogen bond.

graphically in Figures 2 and 3. The carboxylate oxygen atoms (of the 1-carboxy-3-phenylpropyl moiety) bind to the zinc ion in a bidentate fashion, with zinc–oxygen distances of 2.0 and 2.4 Å (Table V). In common with other inhibitors (Weaver et al., 1977; Holmes & Matthews, 1981) the leucyl side chain binds in the $S_1'$ hydrophobic pocket, the primary substrate recognition site of thermolysin. Also, hydrogen bonds between the leucyl carbonyl oxygen and two guanidinium nitrogens, NEE1 and NEE2, of Arg-203 are characteristic of complexes of thermolysin with peptide inhibitors. A hydrogen bond between the NE1 ring nitrogen of tryptophan in the $S_2'$ subsite and the carbonyl oxygen of Asn-111 is exactly as observed with the corresponding tryptophan of phosphoramidon (Weaver et al., 1977). For extended peptide inhibitors studied previously, it has been assumed that the amide nitrogen and the carbonyl oxygen of the $P_2'$ substrate residue can form hydrogen bonds to the OD1 and ND2 atoms of Asn-112, respectively. In the case of the thermolysin–CLT complex, the contact between the $P_2'$ oxygen and ND2 of Asn-112 is 3.1 Å (supporting the first hydrogen bond), but that between the $P_2'$ nitrogen and Asn-112 OD1 is 3.4 Å, suggesting a very weak second hydrogen bond, if one exists at all. Other close contacts between protein and inhibitor are included in Table VI and are discussed in more detail below.

As can be seen in Figure 2, the electron density for the phenyl and the indole rings of the inhibitor is weaker than for other atoms. It appears in Figure 2 that there is almost no density for the two aromatic groups but this is not the case since the lowest electron density contour is drawn at the rather high level of $2.5\sigma$. The variation in the electron density level suggests that the parts of the inhibitor making specific interactions (e.g., hydrogen bonds) with the protein are better

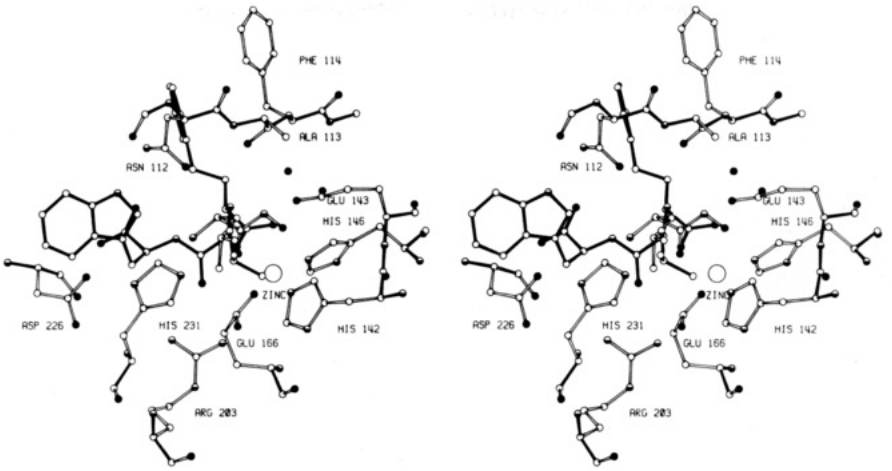


FIGURE 4: Stereo drawing in the vicinity of the zinc ion showing CLT (solid bonds) superimposed on the hydroxamate inhibitor L-Leu-NHOH (open bonds). Carbon atoms are drawn open, oxygen atoms solid, and nitrogen atoms half-solid.

ordered than those parts that make relatively nonspecific (hydrophobic) interactions. This is also supported by the crystallographic refinement (Table IV). The atoms with the highest crystallographic *B* factors are located toward the extremities of the phenyl and the indole rings.

There is very little change in the protein structure on binding the inhibitor. Also, the presence of the bound inhibitor does not appear to substantially perturb the mobility of the residues in the active site.

## Discussion

Perhaps the most interesting aspect of this study is the novel bidentate coordination of the inhibitor carboxylate with the zinc. The oxygens are 2.0 and 2.4 Å from the metal to give overall pentacoordination.

In previous studies, two inhibitors have been observed to bind to thermolysin with a carboxyl group interacting with the zinc. The first such inhibitor is *N*-carbobenzoxy-L-phenylalanine (Cbz-Phe) (Kester & Matthews, 1977b) and the second L-benzylsuccinic acid (Bolognesi & Matthews, 1979). In both cases it appears that the zinc ligation is primarily via a single carboxy oxygen. For L-benzylsuccinic acid, one of the carboxy oxygens is 2.1 Å from the zinc and the second 4.1 Å. Cbz-Phe binds "backward" relative to a normal peptide inhibitor and has somewhat different interactions with the protein (Kester & Matthews, 1977b). In common with CLT, Cbz-Phe has an *N*-carboxymethyl group bound in the $S_1$ subsite, but the alignment of the respective carboxyl groups is different. In particular, one carboxyl oxygen of Cbz-Phe seems to be about 2.1 Å from the zinc and the other 3.0 Å. For both L-benzylsuccinic acid and Cbz-Phe the geometry of binding needs to be confirmed by crystallographic refinement. Presumably the length of the connecting linkage between the carboxyl group and the $\alpha$-carbon in the $S_1'$ subsite is not the critical determinant in monodentate vs. bidentate coordination. Cushman et al. (1977) constructed a series of carboxyalkanoyl inhibitors of the angiotensin converting enzyme and found that the inhibition constant changed monotonically with the increase in length of the acyl chain. No discontinuity was observed in the binding constant that might indicate a change from monodentate to bidentate ligation. It seems reasonable to assume that the bidentate coordination is due to the spatial resemblance between the two oxygens and the nitrogen of the *N*-carboxymethyl group and the two oxygens and the nitrogen of the presumed tetrahedral intermediate during peptide hydrolysis. The concept of the *N*-carboxymethyl group as a mimic of the transition state is explored in more detail in the following paper (Hangauer et al., 1984).

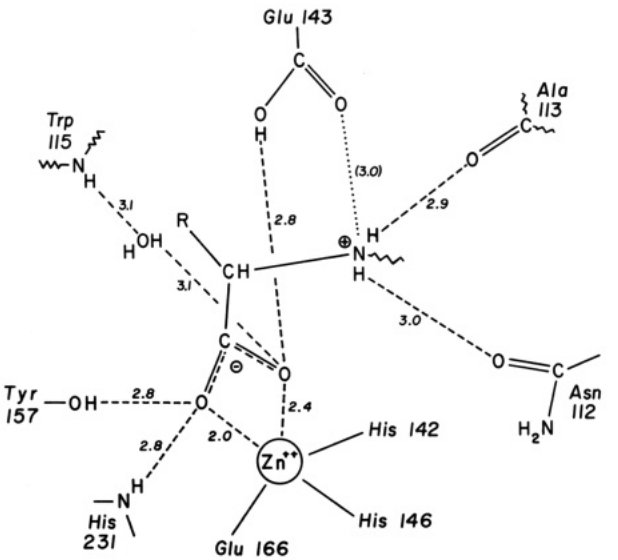


FIGURE 5: Sketch of the apparent interactions and interaction distances between CLT and thermolysin in the vicinity of the zinc ion. Presumed hydrogen bonds are drawn as broken lines, and the close contact between the $\alpha$-amino nitrogen of the inhibitor and Glu-143 is indicated by a dotted line with the distance in parentheses.

Although bidentate zinc coordination in thermolysin has not been seen previously for a carboxylate function, it was observed for hydroxamate inhibitors and led to the suggestion that pentacoordinate zinc complexes might occur as intermediates during catalysis (Holmes & Matthews, 1981). As shown in Figure 4, the positions of the respective zinc-liganding oxygens of CLT and L-Leu-NHOH occupy rather similar positions, reinforcing the idea that pentacoordinate zinc complexes with this geometry may be good models for transition-state intermediates. The respective distances between the two zinc-coordinated oxygens in CLT and in the hydroxamate inhibitor L-Leu-NHOH (Figure 4) are 0.5 and 0.9 Å.

In addition to its interaction with the zinc ion, the carboxylate of CLT participates in a network of hydrogen bonds with the protein (Table VI, Figure 5). One carboxylate oxygen (OG2) is within hydrogen-bonding distance of one oxygen, or possibly both oxygens, of Glu-143 (distances 2.8 and 3.0 Å). Because of this apparent hydrogen bond it appears that one of the participating carboxyls is protonated even though the pH of the crystals is 7.2. In Figure 5 we have drawn the inhibitor carboxyl as unprotonated and the carboxyl of Glu-143 as protonated, although other resonance forms can

be envisaged. When the inhibitor is bound, Glu-143 becomes "buried" and inaccessible to solvent except via the bound water molecule shown in Figure 5, which is 3.2 Å away. The second carboxylate oxygen of the inhibitor (OG1) is hydrogen bonded by both the imidazole nitrogen of His-231 (2.8 Å) and the phenolic hydroxyl of Tyr-157 (2.8 Å). A similar hydrogen bond from His-231 was seen for hydroxamates bound to thermolysin, i.e., in the other example of pentacoordinate zinc ligation. The location of Tyr-157 in the thermolysin active site had suggested it as a candidate for enzyme–substrate interaction (Kester & Matthews, 1977b), but this is the first instance in which this residue is clearly seen to participate in inhibitor binding.

The geometry at the $\alpha$-amino group is also of interest since the nitrogen is located in the thermolysin active site close to the position occupied by the nitrogen of a scissile peptide bond and, as such, provides a model for the tetrahedral nitrogen that is formed during cleavage (see also the following paper). In peptide inhibitors bound to thermolysin previously [e.g., Kester & Matthews (1977b) and Holmes & Matthews (1981)] the NH group at this position has donated a hydrogen bond to the backbone carbonyl oxygen of Ala-113 but has not appeared to participate in other interactions with the protein. With CLT, the situation is somewhat different. In this case, the same hydrogen bond to the peptide backbone at Ala-113 occurs (distance 2.9 Å), but there are two other potential interactions as well, one to the side-chain oxygen of Asn-112 (3.0 Å) and the second to one of the carboxylate oxygens of Glu-143 (3.0 Å). The latter contact is of considerable interest since it suggests the possibility of proton transfer from Glu-143 to the amide nitrogen during catalysis.

Previous discussion of Glu-143 has focused on its role either in the direct nucleophilic attack on the carbonyl carbon of the scissile bond or as a general base, promoting attack on the carbonyl carbon by a water molecule, perhaps in concert with the zinc (Kester & Matthews, 1977b; Holmes & Matthews, 1981). It was suggested that His-231 might act as the proton donor to the departing nitrogen, although it was also pointed out that proton transfer from a water molecule was possible (Kester & Matthews, 1977b). In the present complex, the distance from NE2 of His-231 to the $\alpha$-amino nitrogen is 5.0 Å, too long to suggest direct proton transfer. Detailed stereochemical analysis of possible transition-state intermediates argues against direct proton donation from His-231 (Hangauer et al., 1984). Now the observed binding of CLT to thermolysin suggests an additional possibility, namely, that Glu-143 accepts a proton from the attacking water molecule (as proposed before) and subsequently donates this proton to the leaving nitrogen. In this scenario, in which Glu-143 acts as a "proton shuttle", the role of His-231 would be to stabilize the tetrahedral intermediate.

At the present time there seems to be no definitive way of identifying the proton donor. One approach is to use detailed model building to explore possible transition-state intermediates. This is the subject of the following paper (Hangauer et al., 1984). Another approach is to seek new inhibitors of thermolysin that are as similar as possible to the presumed transition state. In this context we have used crystallographic refinement to improve the accuracy of the complex of thermolysin with phosphoramidon (Weaver et al., 1977) and have determined the mode of binding of *N*-phosphorylleucinamide. Both inhibitors incorporate the tetrahedral phosphoramidate function that we believe to be an excellent mimic of the tetrahedral transition state. These results will be described elsewhere.

It seems reasonable to assume that interactions between *N*-carboxymethyl inhibitors bound to the angiotensin converting enzyme (Patchett et al., 1980) parallel those seen here for thermolysin. Certainly, the successful extension of this inhibitor design from one enzyme to the other (Maycock et al., 1981) supports the assumption that thermolysin can be used as a model for the angiotensin converting enzyme.

*Thermolysin and Carboxypeptidase A.* Carboxypeptidase A is one of the most extensively studied enzymes, yet its mechanism of action remains enigmatic [e.g., see Lipscomb (1982, 1983)]. It is uncertain whether cleavage of peptides and/or esters proceeds via the attack of a water molecule on the carbonyl carbon, promoted by Glu-270 (Lipscomb et al., 1968; Breslow & Wernick, 1977), or by direct nucleophilic attack of the glutamate (Reeke et al., 1967; Makinen et al., 1976, 1982; Cleland, 1977). Most mechanistic proposals invoke Tyr-248 as the proton donor although Mock has suggested that Tyr-248 promotes the nucleophilic attack of a water molecule and that Glu-270 is the proton donor (Mock, 1975; Mock & Chen, 1980).

We have shown previously that there are striking similarities in the three-dimensional arrangement of certain residues in the active sites of thermolysin and carboxypeptidase A (Kester & Matthews, 1977b). The parts of the active site of carboxypeptidase A that have a direct spatial counterpart in the thermolysin are Glu-270, corresponding to Glu-143, and the zinc ion. Also, bound dipeptide or dipeptide analogues occupy closely analogous positions in the active sites of the two enzymes. However, there is no direct counterpart of Tyr-248 in the thermolysin active site. In terms of spatial correspondence, the closest analogue is Asn-112, which has its side-chain oxygen 2.5 Å away from the phenolic oxygen OE of Tyr-248. His-231 of thermolysin has nitrogen NE2 4.3 Å from OE of Tyr-248 [see Figure 3 of Kester & Matthews (1977b)].

On the basis of the close structural correspondence of the zinc ions and the respective glutamates (Glu-270 in carboxypeptidase A; Glu-143 in thermolysin) it was argued that the stereochemical alignment of the glutamate relative to the zinc was vital to catalysis. The lack of spatial correspondence between Tyr-248 of carboxypeptidase A and His-231 of thermolysin was taken to imply that there was no absolute requirement for the involvement of a histidine or a tyrosine in hydrolysis catalyzed by the zinc neutral proteases (Kester & Matthews, 1977b). These inferences are completely consistent with the mechanism for thermolysin suggested by the binding of CLT and elaborated in the following paper (Hangauer et al., 1984). Indeed, the elements of the thermolysin active site that are assumed to be of prime importance for catalysis, namely, the zinc and the glutamate, are the elements also found in the same spatial arrangement in the active site of carboxypeptidase A. This strongly suggests that a mechanism similar to that proposed for thermolysin should also be considered for carboxypeptidase A. In such a mechanism, Glu-270 of carboxypeptidase A would promote the attack of a water molecule on the carbonyl carbon of the substrate, possibly in concert with the zinc via a pentacoordinate complex as has been suggested for thermolysin (Holmes & Matthews, 1981; Kunugi et al., 1982). Glu-270 would then act as a proton shuttle to transfer the proton to the leaving nitrogen, again in analogy with thermolysin (Hangauer et al., 1984). Rather than acting as a proton donor, as has generally been supposed (Lipscomb, 1982, 1983), Tyr-248 of carboxypeptidase A could participate in substrate binding and alignment (at least for peptides) and perhaps help to stabilize the

transition state. Such a mechanism would still permit the other roles envisaged for Tyr-248, for example, as covering the active site, as a recipient of a hydrogen bond for the penultimate peptide bond, as a donor of a hydrogen bond to the newly formed carboxylate anion of product, and in the transfer of substrate along the subsites of carboxypeptidase A (Lipscomb, 1982). It would not be unreasonable that in carboxypeptidase A, an exopeptidase, the flexible Tyr-248 might facilitate translocation of the substrate along the active-site cleft, whereas in thermolysin, an endopeptidase, there would be no translocation of substrates, consistent with the enzyme having a more rigid active-site region.

The mechanism that is suggested herein for peptide cleavage by carboxypeptidase A has elements in common with various mechanisms suggested in the literature but, to our knowledge, has not been proposed before. The role of Glu-270 in promoting the attack of a water molecule has considerable support, but the additional role of Glu-270 as a proton donor (or proton shuttle) has not been suggested. Mock (1975) postulated that Glu-270 might act as a proton donor but proposed a role for Tyr-248 very different to that envisaged here. While we cannot rule out other mechanistic proposals, we believe that the common mechanism that we have proposed for thermolysin and carboxypeptidase A is stereochemically reasonable and would provide a more unified explanation of catalysis by the zinc peptidases than has existed heretofore.

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**Registry No.** CLT, 76400-07-2; thermolysin, 9073-78-3; carboxypeptidase A, 11075-17-5.

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