Inhibition of Thermolysin and Neutral Endopeptidase 24.11 by a Novel Glutaramide Derivative: X-ray Structure Determination of the Thermolysin–Inhibitor Complex†

D. R. Holland,‡ P. L. Barclay,§ J. C. Danilewicz,§ B. W. Matthews,*,‡ and K. James*,§

Institute of Molecular Biology, Howard Hughes Medical Institute and Department of Physics, University of Oregon, Eugene, Oregon 97403, and Pfizer Central Research, Sandwich, Kent, CT13 9NJ, England

Received August 9, 1993; Revised Manuscript Received October 11, 1993●

ABSTRACT: Determination of the X-ray structure of thermolysin–inhibitor complexes has proven useful in aiding our understanding of the mode of binding of inhibitors of related, physiologically important, mammalian zinc peptidases including neutral endopeptidase EC 3.4.24.11 and angiotensin-converting enzyme. Here we describe the mode of binding to crystalline thermolysin of *N*-{1-(2(*R*,*S*)-carboxy-4-phenylbutyl)-cyclopentylcarbonyl}-(*S*)-tryptophan (CCT). CCT is an analogue of both candoxatrilat, a potent inhibitor of neutral endopeptidase 24.11, and of the 5-indanyl ester prodrug candoxatril, which is under clinical evaluation as a potential therapy for congestive heart failure. CCT differs from the previously studied *N*-carboxyalkyl dipeptide CLT [*N*-(*S*)-(1-carboxy-3-phenylpropyl)-(*S*)-leucyl-(*S*)-tryptophan] in several important respects. It has a highly constrained *gem*-cyclopentyl P_1' substituent and lacks the characteristic imino nitrogen substituent of CLT. The structure determination shows that, notwithstanding the conformational influence of the *gem*-cyclopentyl substituent, CCT binds within the active site of thermolysin in a similar manner to CLT. Although the characteristic hydrogen bond between the imino nitrogen of CLT and thermolysin is absent in CCT, the affinities of the two inhibitors for the enzyme are virtually identical. These results illustrate the importance of considering not only those hydrogen bonds that are formed in an enzyme–ligand complex but also the other hydrogen bonds that may be lost due to desolvation of the enzyme and ligand on formation of the complex. In addition, the overall conformational demands placed upon a ligand in order to achieve receptor interaction may be critically important.

Zinc-dependent peptidases play a key role in the biosynthesis and metabolism of a number of bioactive peptides. Consequently, inhibitors of these enzymes have considerable potential as therapeutic agents (Powers & Harper, 1986; Rich, 1990). This potential has been fulfilled in the case of angiotensin-converting enzyme (ACE) inhibitors, which have demonstrated clinical benefit in the treatment of hypertension and congestive heart failure (Patchett & Cordes, 1985; Raia et al., 1990). More recently, inhibitors of neutral endopeptidase EC 3.4.24.11 (endopeptidase 24.11) (Sybertz, 1990; Roques et al., 1990) and matrix metalloproteases (Wahl et al., 1990) are reported to be under investigation as therapies for congestive heart failure and rheumatoid arthritis, respectively.

Our understanding of the hydrolytic mechanism of these physiologically important mammalian peptidases, and their interaction with inhibitors, has been aided greatly by study of the related bacterial endopeptidase thermolysin (TLN), the X-ray crystal structure of which has been reported, both in its native state (Holmes & Matthews, 1982) and in complexes with inhibitors (Matthews, 1988). TLN appears to be a potential model for mammalian enzymes of this class, in particular endopeptidase 24.11, in view of its active site homology (Benchetrit et al., 1988) and the inhibition of both enzymes by the natural product phosphoramidon (**1**) (Suda et al., 1975; Weaver et al., 1977) and the *N*-carboxyalkyl dipeptide inhibitor CLT (**2**) (Maycock et al., 1981; Monzingo & Matthews, 1984) (Figure 1).

N-Carboxyalkyl dipeptides have emerged as an important class of zinc peptidase inhibitors, examples having been reported for TLN (Maycock et al., 1981), endopeptidase 24.11 (Mumford et al., 1982), ACE (Patchett et al., 1980; Patchett & Cordes, 1985), and matrix metalloproteases (Sahoo et al., 1993). A characteristic feature of this inhibitor series is an imino nitrogen substituent, which appears to be involved in hydrogen-bond interactions with the target enzyme. This is supported both by SAR studies, for example, in the enalapril series of ACE inhibitors (Patchett et al., 1980; Patchett & Cordes, 1985), and by X-ray crystallographic studies of CLT in its complex with TLN (Monzingo & Matthews, 1984).

We have reported recently (Danilewicz et al., 1989) a potent and structurally distinct series of inhibitors of endopeptidase 24.11, exemplified by candoxatrilat (**3**), whose 5-indanyl ester prodrug, candoxatril (**4**), is under clinical evaluation as a potential therapy for congestive heart failure. This orally active agent potentiates the actions of atrial natriuretic factor, in animals and man, through inhibition of its metabolism by endopeptidase 24.11. Candoxatrilat is a glutaramide derivative and possesses a number of unusual structural features compared to *N*-carboxyalkyl dipeptides such as CLT. First, it has a highly constrained *gem*-cyclopentyl P_1' substituent, in place of the more common benzyl or isobutyl substituents. Second, it lacks the characteristic imino nitrogen substituent of the *N*-carboxyalkyl dipeptide series, and in fact, introduction of an imino substituent in this series results in a significant reduction in affinity for endopeptidase 24.11 (Danilewicz et al., 1989). In addition, candoxatrilat features a polar methoxyethoxymethyl substituent at P_1, rather than the

† This work was supported in part by grants to B.W.M. from the NIH (GM20066) and the Lucille P. Markey Charitable Trust.

* Authors to whom correspondence should be addressed.
‡ University of Oregon.
§ Pfizer Central Research.

● Abstract published in *Advance ACS Abstracts,* December 1, 1993.

0006-2960/94/0433-51$04.50/0

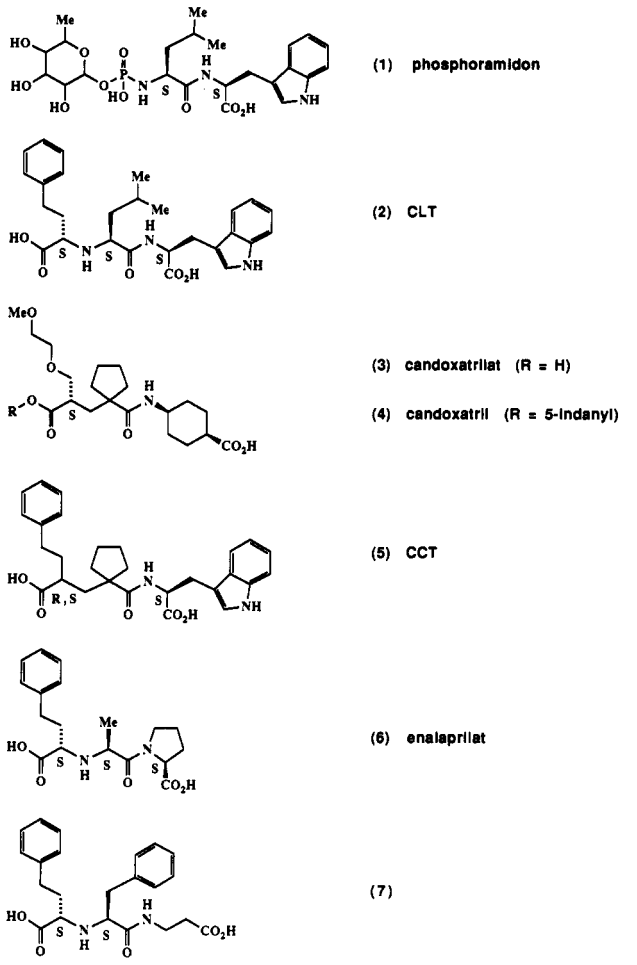

FIGURE 1: Structures of inhibitors described in the text. The structures of thermolysin in complex with phosphoramidon and CLT have been described previously (Weaver & Matthews, 1977; Tronrud et al., 1986; Monzingo & Matthews, 1984). The complex with inhibitor **5** (CCT) is described herein.

lipophilic phenethyl substituent present in CLT and widely employed in ACE inhibitors. Finally, the P_2' substituent of candoxatrilat is the rigid *cis*-4-aminocyclohexanecarboxylic acid.

Given these significant differences in structure between glutaramide inhibitors such as candoxatrilat and *N*-carboxyalkyl dipeptides, it was of considerable interest to us to gain some insight into the mode of interaction of a glutaramide inhibitor with a member of this enzyme class. Since the structure of endopeptidase 24.11 has not been reported, compound **5** (CCT) was designed as an inhibitor of TLN, whose structure, in its complex with the enzyme, could be determined by X-ray crystallography. It incorporates key aspects of the candoxatrilat–glutaramide structure, but functional groups were introduced to facilitate interaction with TLN and allow comparison with the mode of binding of CLT.

Thus, in CCT, the methoxyethoxymethyl P_1 substituent of candoxatrilat has been substituted by the more lipophilic phenethyl group of CLT, a change which is known to be tolerated in the endopeptidase 24.11 inhibitor series (Danilewicz et al., 1989). Further, the rigid *cis*-4-aminocyclohexanecarboxylic acid substituent of candoxatrilat has been replaced with the more flexible (*S*)-tryptophan residue of CLT and phosphoramidon.

We have determined the X-ray crystal structure of CCT bound to TLN and here report the mode of binding of this novel inhibitor type in comparison to the *N*-carboxyalkyl dipeptide, CLT. The structure confirms the general mode of binding that was anticipated and, at the same time, provides new insights into the role of hydrogen bonding (or nonbonding) in enzyme–ligand interaction.

MATERIALS AND METHODS

Enzyme Inhibition Assays. Microvilli were isolated as a brush border membrane fraction from rat kidney according to the method of Booth and Kenny (1974) and used as a source of enzyme for the endopeptidase 24.11 assay. Microvilli (0.041–0.2 mg/mL final concentration) were resuspended in 50 mM HEPES buffer, pH 8.0, containing 150 mM NaCl and 0.6M Na_2SO_4 and preincubated for 15 min at 25 °C in the presence or absence of inhibitors. Endopeptidase 24.11 activity was measured as described previously (Danilewicz et al., 1989), by addition of [glycine-1-^{14}C]hippurylphenylalanylarginine (1–3 mM, specific activity 0.025 mCi/mmol) and incubating at 37 °C for up to 1 h. The reaction was terminated by addition of an equal volume of 1 M HCl, and enzyme activity was assessed by the appearance of radioactive product (i.e., [glycine-1-^{14}C]hippuric acid) extracted into ethyl acetate.

Thermolysin (Calbiochem, final concentration 0.1–0.5 mg/mL) was dissolved in 50 mM HEPES buffer, pH 8.0, containing 150 mM NaCl and preincubated for 15 min at 25 °C in the presence or absence of inhibitors. Thermolysin activity was measured by addition of hippurylphenylalanylarginine (1–3 mM) and incubating at 30 °C for up to 30 min. The reaction was quenched by addition of an equal volume of 0.1 M sodium bicarbonate solution, and the reaction mixture was incubated with a solution of trinitrobenzene sulphonate (final concentration 15 mM) for 3 min at 25 °C. The resultant yellow solution, which develops as a result of the reaction between the trinitrobenzene sulfonate and free amino terminus of the phenylalanylarginine product was quantified by measuring absorbance at 414 nM wavelength.

Molecular Modeling. Molecular modeling used CHEMLAB (Pearlstein, 1982) software to estimate the intramolecular energy differences between conformers of CLT and CCT. Models of the two inhibitors were "built" starting with the X-ray coordinates for the enzyme-bound conformation of CLT, and X-ray crystal conformation of an analogue of CCT, and then protonating both the carboxyl groups and also the imino group of CLT. Charges on atoms were calculated by CNDO/2. Standard CHEMLAB parameters were used to estimate electrostatic (dielectric constant set to 4.0), van der Waals nonbonded attraction and repulsion, and torsional effects.

Energy maps (Ramachandran maps) were derived by rotating pairs of torsional angles through 360° in 30° increments, using the SCAN routine of CHEMLAB. No relaxation of bonds or angles was invoked.

Crystallography. Thermolysin (Calbiochem) was crystallized (Holmes & Matthews, 1982) and stored in a mother liquor (M7) comprised of 0.01 M calcium acetate, 0.01 M Tris-acetate, and 7% (v/v) dimethyl sulfoxide, pH 7.25. Optimal conditions for binding of the inhibitor were determined by soaking native crystals in M7 containing increasing amounts of inhibitor for several days and calculating (*h0l*) difference Fourier projections between native and inhibitor complex (Weaver et al., 1977).

Maximum binding was obtained by slowly equilibrating a thermolysin crystal to a final inhibitor concentration of 5 mM and soaking for a total of 5 days. A single crystal was used to collect three-dimensional data to 1.7-Å resolution on a Xuong-Hamlin area detector (Xuong, 1985) using graphite-

Table 1: Data Collection and Refinement Statistics[a]

data collection	
resolution (Å)	1.6
number of independent reflections	34103
completeness (%)	88.7
R_{merge} (%)	6.6
average isomorphous difference (%)	14
refinement	
resolution limits (Å)	10.0–1.7
number of reflections	33922
initial R value (%)	24.6
final R value (%)	16.2
number of refinement cycles	20
number of atoms	2623
Δ(bond length)	0.017
Δ(bond angle)	2.5

[a] R_{merge} is the discrepancy between repeated intensity measurements. The "average isomorphous difference" is the average difference between structure amplitudes observed for the native thermolysin structure and the thermolysin–inhibitor complex. Δ(bond length) and Δ(bond angle) give the average discrepancies of the bond lengths and bond angles in the final refined model relative to "ideal" stereochemistry.

Table 2: Comparison of the Inhibition of Endopeptidase 24.11 and Thermolysin[a]

	enzyme	
inhibitor	endopeptidase 24.11 K_i (μM)	thermolysin K_i (μM)
phosphoramidon	0.020	0.08
CLT	0.969	0.56
candoxatrilat	0.018	2.21
CCT	0.071	0.38

[a] Inhibition of enzyme activity was measured as described under Materials and Methods. The inhibition constant (K_i) was determined using the method of either Dixon (1953) or Cheng and Prussof (1973). Data represent the average of 2–15 determinations.

monochromated Cu Kα radiation from a Rigaku RU200 rotating anode generator. Intensity statistics are given in Table 1.

An initial electron density map (Figure 2) was calculated with amplitudes ($F_{PI} - F_c$) and phases ϕ_c where F_{PI} is the observed structure amplitude for the thermolysin–inhibitor complex and F_c and ϕ_c were calculated from the refined model of the wild-type structure (Holmes & Matthews, 1982) with solvent molecules removed from the active-site cleft [cf. Monzingo and Matthews (1984)]. This map clearly showed the overall alignment of the bound inhibitor, although the density was weak for some atoms in the phenyl and indole rings.

The protein–inhibitor complex was refined using the TNT package (Tronrud et al., 1987; Tronrud, 1992). Both thermal factors and coordinates were refined simultaneously, starting with loose weights and later increasing the constraints to expected stereochemistry. Atoms that were not clearly visible in the initial ($F_{PI} - F_c$) difference map were not included in the initial model. After 10 cycles of refinement, these atoms could be seen in maps with coefficients ($2F_{PI} - F_c$) and were added to the model. Refinement statistics are included in Table 1.

RESULTS

The inhibitory potencies of phosphoramidon, CLT, candoxatrilat, and CCT *versus* endopeptidase 24.11 and TLN are given in Table 2. As described by Maycock et al. (1981), CLT is an inhibitor of TLN. It is also a relatively weak inhibitor of endopeptidase 24.11, being almost two orders of magnitude less potent than candoxatrilat. Candoxatrilat is one of the most potent inhibitors of endopeptidase 24.11 reported to date (Barclay, 1990). Its high affinity (Table 2) may be a consequence of preorganization into an enzyme-bound conformation, resulting from the conformational influences of both the geminal-cycloalkyl P_1 substituent and the rigid *cis*-4-aminocyclohexanecarboxylic acid P_2' substituent. Notably, it is some two orders of magnitude selective for inhibition of endopeptidase 24.11 over thermolysin.

Replacement of the methoxyethoxymethyl P_1 side chain of candoxatrilat with phenethyl, and the highly constrained *cis*-4-aminocyclohexanecarboxylic acid P_2' substituent with (*S*)-tryptophan, yields inhibitor **5** (CCT). This modification results in a decrease in affinity *versus* endopeptidase 24.11 but increases affinity for thermolysin. In fact, **5** appears equipotent with CLT as an inhibitor of thermolysin, despite being a diastereoisomer mixture, and lacking an imino nitrogen substituent, and is a superior inhibitor of endopeptidase 24.11. The phosphoryl dipeptide, phosphoramidon (**1**), was the most potent inhibitor tested and appears to represent a good mimic of the transition state for peptide bond hydrolysis by these enzymes (Weaver et al., 1977; Tronrud et al., 1986).

In the crystallographic analysis, the electron density for the bound CCT is sufficiently well defined (Figure 2) to permit assignment of the configuration of the bound diastereoisomer as (*S*,*S*) rather than (*R*,*S*). This would be consistent with the (*S*,*S*,*S*)-diastereoisomer of CLT being the tightest binder to TLN (Maycock et al., 1981), and the (*S*)-isomer at the P_1 position being the more potent isomer in both the ACE inhibitor (Patchett et al., 1980; Patchett & Cordes, 1985) and endopeptidase 24.11 inhibitor (Danilewicz, 1989) series. In contrast, the electron density for the indole ring of the inhibitor is weak, suggesting that this part of the inhibitor is mobile or exists in two or more alternative conformations. This has been noted previously for related inhibitors bound to TLN (Weaver et al., 1977; Monzingo & Matthews, 1984).

Figure 3 provides a (stereo)representation of the bound inhibitor (*S*,*S*)-CCT in the thermolysin active site, and Figure 4 illustrates the bound inhibitor superimposed upon the coordinates of CLT (Monzingo & Matthews, 1984). It is apparent that the two molecules superimpose very closely, adopting very similar enzyme-bound conformations. The only significant deviation in conformation appears to be in the region of the indole ring. Because of the aforementioned uncertainty in the density of this ring, it is not clear whether this difference is real. Most likely, both inhibitors are more-or-less equally mobile or disordered in this region, and the refined coordinates correspond to representative conformers populated in each case.

Figure 5 is a schematic representation illustrating some of the contact distances between (*S*,*S*)-CCT and the enzyme. These distances are given in more detail in Table 3 and compared with the corresponding distances for CLT. The inhibitor makes a large number of contacts with the enzyme which were anticipated from its structural similarity to CLT. In particular, there are apparent hydrogen bonds between the inhibitor amide carbonyl and side chain of Arg 203, the tryptophan carboxylate of the inhibitor and sidechain NH_2 of Asn 112, and the tryptophan NH of the inhibitor and main chain carbonyl of Ala 111. The *gem*-cyclopentyl ring binds into a lipophilic region of the enzyme in a manner analogous to that of the isobutyl group of leucine-containing inhibitors. The second inhibitor carboxyl group makes a series of interactions with the zinc ion, Glu 166, His 231, and the catalytic Glu 143. The phenethyl P_1 chain of the inhibitor occupies a second lipophilic site in the region of Phe 114.

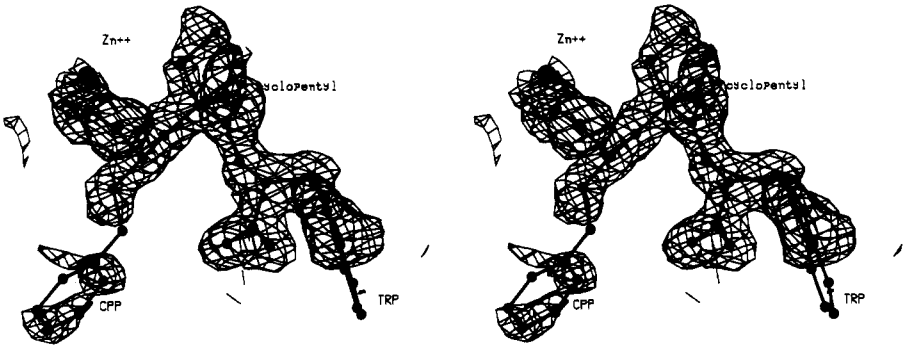

FIGURE 2: Initial map showing the electron density corresponding to bound CCT; coefficients ($F_{PI} - F_c$) where F_{PI} is the observed structure amplitude of the protein–inhibitor complex and F_c is the amplitude calculated from the refined model of thermolysin (Holmes & Matthews, 1982) with solvent molecules and dipeptides removed from the active site region. Phases, ϕ_c, correspond to amplitudes, F_c. Map contoured at 3.0σ where σ is the root-mean-square density throughout the unit cell. Coordinates of the refined inhibitor structure are superimposed.

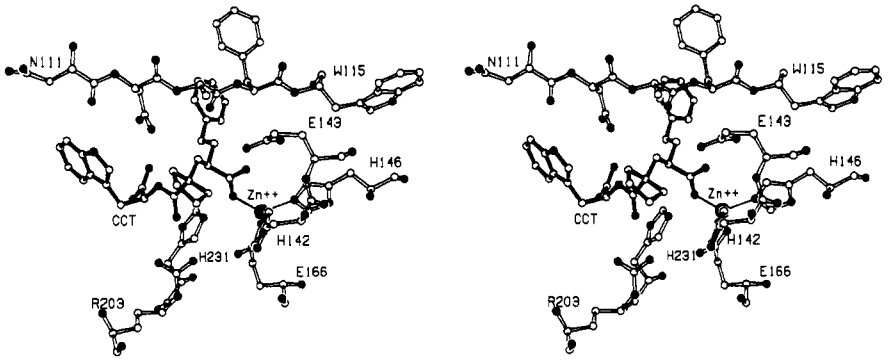

FIGURE 3: Refined model showing the binding of CCT to thermolysin. The direction of view is from the center of the enzyme looking "through" the inhibitor and toward the surface of the enzyme. Oxygen atoms are drawn solid, nitrogen atoms half solid, and carbon atoms are shown as open circles. The bonds of the inhibitor are drawn solid; those of the protein are open. Interactions with the zinc ion are shown as thin lines.

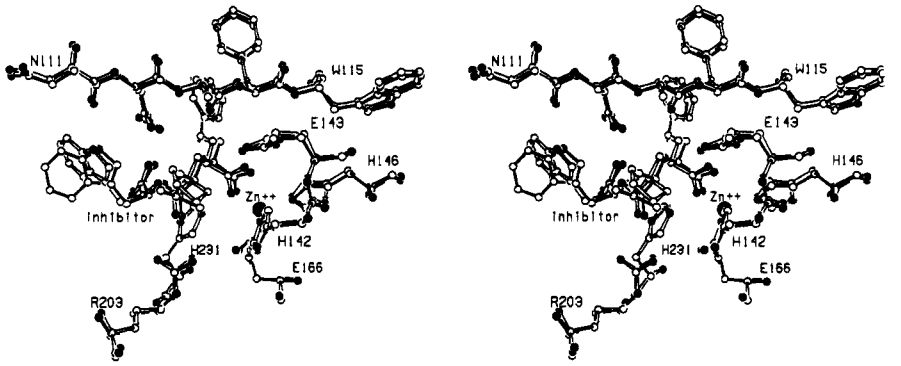

FIGURE 4: Refined coordinates of the CCT–thermolysin complex (open bonds) superimposed on those of the CLT–thermolysin complex (solid bonds). Conventions to show different atom types are as in Figure 3.

DISCUSSION

Neutral endopeptidase 24.11 and TLN are considered closely related in their mechanism of peptide bond hydrolysis and their active site topography (Benchetrit et al., 1988). Thus, the active site sequence homology between these enzymes has been pointed out (Benchetrit et al., 1988), including, in particular, the presence in the endopeptidase 24.11 sequence of residues corresponding to the zinc ligands in TLN, the catalytic glutamic acid, and even specific amino acid residues in a β-sheet region comprising the active site of TLN. Furthermore, inhibitors have been reported which show high affinity for both enzymes, for example, the transition-state mimic phosphoramidon (**1**) [*N*-(rhamnosylphosphoryl)leucyltryptophan]. The structure of the complex between phosphoramidon and TLN has been determined (Weaver et al., 1977; Tronrud et al. 1986), and highlights specific interactions between the leucyltryptophan dipeptide moiety of the inhibitor and the active site residues of TLN which contribute to its high enzyme affinity.

Subsequently, this dipeptide moiety was employed by Patchett et al. (1980) as the basis for a carboxyalkyl dipeptide inhibitor of thermolysin, CLT (**2**). This agent represented a further extension of the zinc peptidase-inhibitor design principle which had been devised originally for inhibitors of ACE but has subsequently found utility in inhibitors of endopeptidase 24.11 and matrix metalloproteases. Thus, CLT is related in structure to the ACE inhibitor enalaprilat (**6**) and the neutral endopeptidase 24.11 inhibitor (**7**). The structure of the complex between CLT and TLN revealed a series of specific interactions between the leucyltryptophan moiety of the inhibitor and the enzyme: in particular, occupation of the lipophilic P_1' pocket of TLN by the isobutyl

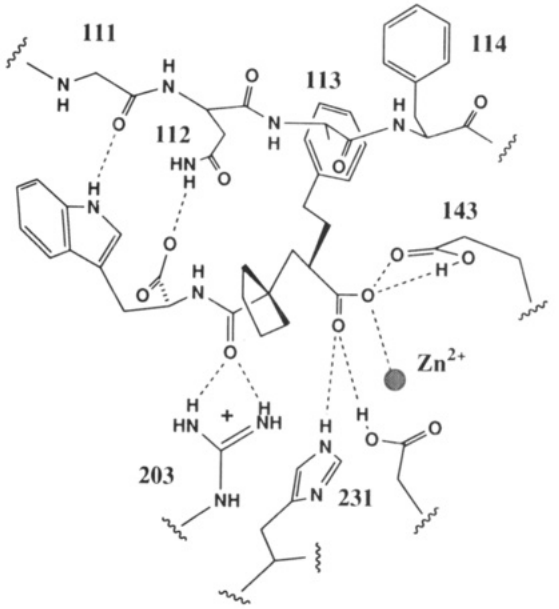

FIGURE 5: Schematic drawing showing hydrogen bonds (dashed lines).

Table 3: Comparison of Thermolysin–Inhibitor Contacts for CCT and CLT[a]

TLN residue	inhibitor atom (CCT and [CLT])	TLN–CCT distance	TLN–CLT distance[b]
O–Asn 112	CH_2 (backbone) [NH, backbone]	3.2	3.2 (H)
O–Asn 111	$N^{\epsilon 1}$–Trp (indole)	3.0 (H)	2.9 (H)
$N^{\delta 2}$–Asn 112	O–Trp (carboxyl)	3.2 (H)	3.1 (H)
O^{δ}–Asn 112	NH–Trp (backbone)	3.5	3.4 (H)
O–Ala 113	CH_2 (phenpropyl)	3.1	3.2
O–Ala 113	CH_2 (backbone) [NH, backbone]	3.1	2.9 (H)
$N^{\epsilon 2}$–His 142	O–2 (carboxyl)	3.4	3.2
$O^{\epsilon 1}$–Glu 143	O–1 (carboxyl)	3.2 (H)	2.8
$O^{\epsilon 2}$–Glu 143	CH_2 (backbone) [NH, backbone]	3.4	3.1
$O^{\epsilon 2}$–Glu 143	O–1 (carboxyl)	2.9 (H)	3.0 (H)
$O^{\epsilon 1}$–Glu 166	O–2 (carboxyl)	3.0 (H)	2.8 (H)
$N^{\epsilon 1}$–Arg 203	O (amide)	2.9 (H)	2.9 (H)
$N^{\epsilon 2}$–Arg 203	O (amide)	2.9 (H)	2.9 (H)
$N^{\epsilon 2}$–His 231	O–2 (carboxyl)	2.8 (H)	2.8 (H)
Zn^{2+}	O–2 (carboxyl)	2.0	2.0
water	O–1 (carboxyl)	3.1 (H)	3.1 (H)
water	O–Trp (carboxyl)	2.8 (H)	

[a] The table compares inhibitor–enzyme interactions for both CCT and CLT. Where the inhibitors differ in structure, the atom type in CLT is given in square brackets. The symbol (H) denotes a presumed hydrogen bond. [b] From Monzingo and Matthews (1984).

side chain of leucine; hydrogen bonds between the leucyl carbonyl and Arg 203; hydrogen bonds between the tryptophan carboxyl and Asn 112; a hydrogen bond between the tryptophan indole NH and the carbonyl of Ala 111. In addition, the carboxyphenpropyl group of the inhibitor provides both a strong ligand for the zinc ion and a lipophilic interaction with the P_1 pocket of the enzyme. An important observation is the hydrogen bond between the leucine imino nitrogen of the inhibitor and the carboxyl group of the catalytic Glu 143 of the enzyme. This observation is consistent with SAR findings in the ACE inhibitor *N*-carboxyalkyl dipeptide series, where the imino substituent is crucial for high enzyme affinity (Patchett et al., 1980; Patchett & Cordes, 1985). The complex between CLT and TLN is therefore considered to represent the likely mode of interaction between *N*-carboxyalkyl dipeptides and ACE (Monzingo & Matthews, 1984) and, by analogy, their mode of interaction with related enzymes such as endopeptidase 24.11.

CCT is an inhibitor of both endopeptidase 24.11 and TLN. Introduction of the (*S*)-tryptophan residue has reduced affinity for endopeptidase 24.11 compared to candoxatrilat, but it is nevertheless a better inhibitor of endopeptidase 24.11 than CLT. Affinity for TLN has been increased over candoxatrilat by this maneuver, and CCT is of potency similar to that of CLT as a TLN inhibitor. CCT therefore represents an excellent tool with which to study the mode of interaction of a glutaramide inhibitor to TLN as a representative of this enzyme class. We were confident, in view of the relative enzyme affinities of CCT and CLT toward TLN, that the modes of interaction of the two inhibitors should be very similar, as is shown to be the case.

A notable difference, however, is that the CCT structure precludes hydrogen bond interactions corresponding to those made by the imino substituent of CLT, which is lacking in this inhibitor. Thus, the central methylene group of the inhibitor is in close contact with the carbonyl of Ala 113 (3.1 Å), the side chain carbonyl group of Asn 112 (3.2 Å), and one of the side chain oxygens of Glu 143 (3.4 Å). However, for CCT, these are all non-hydrogen-bonding interactions (Table 3). Consequently, the hydrogen-bonding potential of the carbonyl of Ala 113 is left completely unsatisfied, a situation which, at first sight, would appear to be energetically unfavorable and destabilizing to the complex. Despite the fact that this network of hydrogen bond interactions is missing in the case of the glutaramide inhibitor, the enzyme-bound conformation of CCT matches very closely that of CLT, and furthermore, the affinity of these two inhibitors for TLN is almost identical.

This situation is analogous to that found by Bartlett et al. (1991), in which two even more closely related phosphorus-containing inhibitors of TLN, differing only by substitution of a methylene group for an imino group, have very similar affinities for the enzyme. In this latter case, it was possible to conclude, because of the extremely close similarity of the two inhibitors, that the net binding contribution of the methylene and imino substituents was the same. This was rationalized on the basis that although the imino-substituted inhibitor forms a series of hydrogen bond interactions in the enzyme–inhibitor complex, it loses an equal number of hydrogen bond interactions with solvent in doing so (Fersht, 1987; Bartlett et al., 1991). Although the methylene group makes no hydrogen bonds in the complex, neither does it lose any to solvent on binding to the enzyme. Thus, the net binding contribution of these two quite different functional groups is roughly the same. Stated another way, while it is favorable to transfer the hydrophobic methylene group from solvent to a buried site in the protein, it is also unfavorable because it requires the desolvation of protein hydrogen bond acceptors at that site.

It is possible that a similar argument holds for the interaction of CCT with thermolysin in comparison with CLT. Thus, although CCT contributes no hydrogen bonds to the enzyme *via* its methylene group, neither does it lose any hydrogen bonds to solvent on complex formation. However, unlike the situation described by Bartlett et al. (1991), the comparison between CCT and CLT must also take into account the effects on inhibitor conformation of substituting a *gem*-cyclopentyl for an isobutyl group. Thus, we have undertaken a conformational analysis of the CCT structure in comparison with that of CLT. The X-ray crystallographic structure of a crystalline glutaramide analogue of CCT was employed as a

starting point for the analysis and proved to be very close to the global energy minimum. Adjustment of this conformation to match the TLN-bound conformation of CCT required only a small energy expenditure (*ca.* 0.7 kcal mol^{-1}). In contrast, conformational analysis of the CLT system revealed that its TLN-bound conformation was 3.0 kcal mol^{-1} above its global energy minimum. Thus, an alternative explanation of the equivalent enzyme affinity of CCT and CLT, despite the difference in hydrogen bond inventory in each case, is that while CLT derives binding energy *via* hydrogen bonds from the imino nitrogen group to the enzyme, this is offset by the cost of adopting the binding conformation. In the case of CCT, although it derives no binding energy from hydrogen bonds *via* its methylene group, neither does it pay a significant energy cost in adopting the binding conformation.

In conclusion, study of the interaction between a novel zinc peptidase inhibitor type and thermolysin has once again provided insight into the mode of interaction of these compounds with this enzyme class and highlighted the subtle factors that can govern enzyme affinity. The experimental results presented here may provide a useful case with which to test theoretical methods for studying flexibility and desolvation effects in ligand binding.

ACKNOWLEDGMENT

We thank Mrs. Lisa Reed and Mr. Paul Raymond for technical assistance in carrying out enzyme assays, Dr. Steven Roderick for helpful advice regarding crystallographic procedures, and Dr. Michael S. Tute for undertaking molecular modeling studies.

REFERENCES

Barclay, P. L., Danilewicz, J. C., & Samuels, G. M. R. S. (1990) *Lancet*, 754–755.

Bartlett, P. A., Morgan, B. P., Scholtz, J. M., Ballinger, M. D., & Zipkin, I. D. (1991) *J. Am. Chem. Soc. 113*, 297–303.

Benchetrit, T., Bissery, V., Mornon, J. P., Devault, A., Crine, P., & Roques, B. P. (1988) *Biochemistry 27*, 592–596.

Booth, A. G., & Kenny, A. J. (1974) *Biochem. J. 142*, 575–581.

Cheng, Y., & Prussof, W. H. (1973) *Biochem. Pharmacol. 22*, 3099–3108.

Danilewicz, J. C., Barclay, P. L., Barnish, I. T., Brown, D., Campbell, S. F., James, K., Samuels, G. M. R. S., Terrett, N. K., & Wythes, M. J. (1989) *Biochem. Biophys. Res. Commun. 164*, 58–65.

Dixon, M. (1953) *Biochem. J. 55*, 170–171.

Erdos, E. G., & Skidgel, R. A. (1989) *FASEB J. 3*, 145–151.

Fersht, A. R. (1986) *Trends Biochem. Sci. 12*, 301–304.

Hangauer, D. G., Monzingo, A. F., & Matthews, B. W. (1984) *Biochemistry 23*, 5730–5741.

Holmes, M. A., & Matthews, B. W. (1982) *J. Mol. Biol. 160*, 623–629.

Matthews, B. W. (1988) *Acc. Chem. Res. 21*, 333–340.

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.

Monzingo, A. F., & Matthews, B. W. (1984) *Biochemistry 23*, 5724–5729.

Mumford, R. A., Zimmerman, M., ten Broeke, J., Taub, D., Joshua, H., Rothrock, J. W., Hirshfield, J. M., Springer, J. P., & Patchett, A. A. (1982) *Biochem. Biophys. Res. Commun. 109*, 1303–1309.

Patchett, A. A., & Cordes, E. H. (1985) *Adv. Enzymol. Relat. Areas Mol. Biol. 57*, 1–84.

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, R. M., & Hirschmann, R. (1980) *Nature 288*, 280–283.

Pearlstein, R. A. (1982) Ph.D. Dissertation, Case Western Reserve University, Cleveland, OH.

Powers, J. C., & Harper, W. J. (1986) *Proteinase Inhibitors* (Barrett, A. J., & Salvesen, G., Eds.) p 219, Elsevier, Amsterdam.

Raia, J. J., Barone, J. A., Byerly, W. G., & Lacey, C. R. (1990) *Ann. Pharmacother. 24*, 506–525.

Rich, D. H. (1990) *Comprehensive Medicinal Chemistry* (Hansch, C., Sammes, P. G., & Taylor, J. B., Eds.) Vol. 2, pp 391–436, Pergamon, Oxford.

Roques, B. P., & Beaumont, A. (1990) *Trends Pharmacol. Sci. 11*, 245.

Sahoo, S. P., Polo, S. A., Durette, P. L., Esser, C. K., Hagmann, W. K., Kopka, I. E., Chapman, K. T., & Caldwell, C. G. (1993) PCT Int. Appl. WO9221360-A1, *Chem. Abstr. 118(23)*, 234493v.

Suda, H., Aoyagi, T., Takeuchi, T., & Umezawa, H. (1973) *J. Antiobiot. 26*, 621–623.

Sybertz, E. J. (1990) *Cardiovasc. Drug Rev. 8*, 71.

Tronrud, D. E. (1992) *Acta Crystallogr. A48*, 912–916.

Tronrud, D. E., Monzingo, A. F., & Matthews, B. W. (1986) *Eur. J. Biochem. 157*, 261–268.

Tronrud, D. E., Ten Eyck, L. F., & Matthews, B. W. (1987) *Acta Crystallogr. A43*, 489–503.

Wahl, R. C., Dunlap, R. P., & Morgan, B. A. (1990) *Annu. Rep. Med. Chem. 25*, 177–184.

Weaver, L. H., Kester, W. R., & Matthews, B. W. (1977) *J. Mol. Biol. 114*, 119–132.

Xuong, N. H., Nielsen, C., Hamlin, R., & Anderson, D. (1985) *J. Appl. Crystallogr. 181*, 342–350.