Peptide Hydroxamic Acids as Inhibitors of Thermolysin†

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ABSTRACT: A series of potent and specific inhibitors for the zinc metalloendoprotease thermolysin have been developed. Peptides which contained the thiosemicarbazide (-NHN(R)CS-) functional group and which were structurally related to good thermolysin substrates were first synthesized and examined. No inhibition was observed even though the sulfur atom of the thiosemicarbazide was expected to coordinate to the zinc atom of thermolysin. Peptide hydrazides were shown to be weak inhibitors of thermolysin with K_i values in the millimolar range. Subsequently, a peptide hydroxamic acid, Z-Gly-L-Leu-NHOH, was found to be a remarkably strong inhibitor with a K_i value of 13 μM at pH 7.2. Studies on the role of the hydroxamic acid functional group, on the stereospecificity, the effect of peptide chain length, and the side-chain specificity were carried out using a number of analogous peptide hydroxamic acids. The results indicate that the N oxygen of the hydroxamic acid interacts with the zinc atom at the catalytic site of thermolysin, while the bulky side chain of leucine is accommodated at the hydrophobic S_1' binding site of the enzyme. The preference for a L-leucyl residue at this binding site is 72 times greater than a glycyl residue. Elongation of the peptide chain decreased the inhibitory activity of the hydroxamic acids, indicating the lack of favorable interactions between the peptide chain of the inhibitor and the extended substrate binding site of thermolysin. Incorporation of a benzylmalonylhydroxamic acid moiety into a peptide structure resulted in a distinct improvement in inhibition. One of the best inhibitors in the series was HONH-Bzm-L-Ala-Gly-NH_2 (Bzm = $-COCH(CH_2C_6H_5)CO-$) which had a K_i value of 0.66 μM. This was 30 times better than HONH-Bzm-OEt, indicating that it interacted with thermolysin's extended substrate-binding region. Other metalloproteases such as leucine aminopeptidase, carboxypeptidase A, and *Clostridium histolyticum* collagenase and the serine protease chymotrypsin were not inhibited by HONH-Bzm-L-Ala-Gly-NH_2. The results are consistent with a model for inhibitor binding involving coordination of the hydroxamic acid functional group to the zinc atom of the enzyme, interaction of the benzyl group with the primary specificity site (S_1'), and interaction of the alanyl moiety with S_2' subsite of thermolysin.

Metalloendoproteases are involved in many important physiological processes. For example, collagenase is thought to be the destructive agent in arthritis (Harris and Krane, 1974), and the angiotensin-converting enzyme is an important element in the regulation of blood pressure (Peach, 1977). These enzymes are characterized by the presence of an essential zinc atom and are inhibited by chelating reagents, such as ethylenediaminetetraacetic acid and 1,10-phenanthroline, and by thiol compounds, such as cysteine. Specific and potent inhibitors would be useful for the regulation of the enzyme activity of metalloproteases involved in a variety of diseases and physiological processes. We have attempted to design inhibitors which would be directed to both catalytic and binding sites of specific metalloproteases in order to achieve the desired specificity and potency.

Thermolysin, a widely studied metalloendoprotease of bacterial origin, was used as the model system in initial experiments, since detailed structural information was available for this enzyme. This enzyme hydrolyzes the peptide bonds at the imino group of hydrophobic amino acid residues, such as leucine, isoleucine, and phenylalanine (Morihara, 1967; Morihara and Oka, 1968; Morihara et al., 1968); a typical synthetic substrate is Z-Gly-L-Leu-NH_2.[1] X-ray crystallographic studies have shown that the catalytic site of thermolysin contains a zinc atom to coordinate and polarize the scissile peptide bond carbonyl oxygen of a substrate. During hydrolysis, Glu-143 promotes the nucleophilic attack of a water molecule upon the carbonyl carbon of the scissile peptide bond, and His-231 donates a proton to the peptide nitrogen (Kester and Matthews, 1977). The primary binding site (S_1')[2] is a hydrophobic pocket consisting of the bulky side chains of Leu-202 and Val-139.

In addition to chelating reagents, inhibitors of thermolysin include dipeptide derivatives which have the reverse sequence (e.g., Z-L-Leu-Gly-NH_2) of typical substrates (Feder et al., 1974). Phosphoramidon (Rhamnosyl-P-L-Leu-L-Trp-OH) and several phosphoryl peptides are extremely potent inhibitors of bacterial neutral proteases (Suda et al., 1973). The present study started with simple combination of a chelating moiety and a leucyl residue into a structure which would be expected to bind to the enzyme in a manner analogous to the binding of substrates to thermolysin. In this paper, we report the syntheses and the inhibitory activities of a series of peptides containing thiocarbazyl (-NHN(R)CS-) or carbazyl functional groups, peptide hydrazides, and peptide hydroxamic acids.

Materials and Methods

Thermolysin (three times crystallized) was purchased from Calbiochem (San Diego). Furylacryloyl-Gly-L-Leu-NH_2 was obtained from Vega-Fox Biochemicals (Tucson, Arizona). *N,N'*-Dicyclohexylcarbodiimide, *N*-hydroxybenzotriazole,

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[1] Abbreviations used: Agly, azaglycyl, -NHNHCO-; Aala, azaalanyl, $-NHN(CH_3)CO-$; Aphe, azaphenylalanyl, $-NHN(CH_2C_6H_5)CO-$; Bzm, benzylmalonyl, $-COCH(CH_2C_6H_5)CO-$; NA, *p*-nitroanilide; Z, benzyloxycarbonyl; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

[2] The nomenclature used for the individual amino acid residues (P_1, P_1', P_2', etc.) of an inhibitor and for the subsites (S_1, S_1', S_2', etc.) of the enzyme is that of Schechter and Berger (1967).

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TABLE I: Inhibition of Thermolysin by Peptide Hydrazides.[a]

Hydrazide	K_i (mM)
Z-NHNH-CS-Leu-$NHNH_2$	6.7
Z-Agly-Leu-$NHNH_2$	0.38
Z-Gly-Leu-$NHNH_2$	1.1
Ac-Ala-Aphe-Leu-$NHNH_2$	6.5
Ac-Ala-Ala-Aala-Leu-$NHNH_2$	7.9

[a] 0.1 M Tris-HCl (pH 7.2), 2% dimethylformamide, 25 °C.

N-methyl- and *O*-benzylhydroxylamine hydrochlorides, and diethyl benzylmalonate were the products of Aldrich Chemical Co., Inc. *N*-Hydroxysuccinimide was obtained from Eastman Kodak Co. *O*-Methylhydroxylamine hydrochloride was obtained from Pfartz and Bauer, Inc. L-Leucinehydroxamic acid, chymotrypsin A_α, carboxypeptidase A, leucine aminopeptidase, and *Clostridium histolyticum* collagenase were purchased from Sigma Chemical Co. The synthesis of all new compounds are described in the supplementary material.

Assay Method. Thermolysin activities with or without inhibitors were measured using furylacryloyl-Gly-L-Leu-NH_2 as substrate (Walsh et al., 1974). To 2 mL of substrate solution in 0.1 M Tris-HCl (pH 7.2) containing 2% dimethylformamide was added 0.1 mL of 8.3 μM enzyme solution in 0.1 M Tris-HCl, 0.01 M $CaCl_2$ (pH 7.2). The decrease in the absorbance at 345 nm was followed with a Beckman Model 25 spectrophotometer at 25 °C. K_i values of the inhibitors were determined from Dixon plots employing 1 and 2 mM substrate concentrations. Under the conditions of the furylacryloyl-Gly-L-Leu-NH_2 assay ($K_M \gg$ [S]), Dixon plots do not distinguish between competitive and noncompetitive inhibition. The K_i values of two of the best inhibitors (Z-Gly-L-Leu-NHOH and HONH-Bzm-L-Ala-Gly-NH_2) were checked by means of Henderson plots (Henderson, 1972).

Results

Thiocarbazate and Hydrazides. Thiocarbazates such as Z-NHNH-CS-L-Leu-OEt were the first compounds which we designed and tested as possible thermolysin inhibitors. These compounds are structurally related to good thermolysin substrates such as Z-Gly-L-Leu-NH_2 but have a sulfur atom in place of the oxygen atom of the scissile peptide bond and have a NH in place of the CH_2 group of the glycyl residue. The latter modification was made in order to have a structure with a reasonable possibility of synthesis.

Though the peptide thiocarbazates were expected to bind to thermolysin via sulfur–zinc coordination which would result in inhibition of enzyme activity, Z-NHNH-CS-L-Leu-OEt showed no inhibitory activity toward thermolysin. Possibly the sulfur atom of the thiosemicarbazide is not electronegative enough to coordinate to the zinc of thermolysin or alternately the sulfur may be too large compared to oxygen to allow proper binding of the inhibitor. On the other hand, the hydrazide, Z-NHNH-CS-L-Leu-$NHNH_2$ appeared to be a weak inhibitor of thermolysin. Other peptide hydrazides were synthesized and shown to weakly inhibit thermolysin activity with K_i values of about 1 mM (Table I). The elongated peptide chains which were expected to make some contact with the extended binding site of the enzyme to enhance the binding gave no decrease in K_i values. The protection of the hydrazide group by reaction with ethyl chloroformate caused a complete loss of inhibitory activity, indicating that the hydrazide nitrogen plays an important role in inhibition.

Hydroxamic Acids. From the consideration that the oxygen of the hydroxamic acid functional group (-CONHOH) is a better electron donor to a metal ion than the nitrogen atom of a hydrazide (-$CONHNH_2$), the hydroxamic acid moiety was introduced into the same sequence. Thus, Z-Agly-L-Leu-NHOH appeared to be a remarkably good inhibitor with a K_i value of 2.7 μM. This compound was unfortunately, however, unstable in aqueous buffer at pH 7.2. A 24-h-old solution in dimethylformamide maintained full activity but a buffer solution lost the activity with half-lives of 6 and 24 h at 25 and 4 °C, respectively. This is probably due to an internal cyclization reaction involving the hydroxamic acid moiety which cleaves the molecule at the azaglycyl residue. Therefore, the simple hydroxamic acid, Z-Gly-L-Leu-NHOH, whose structure is very close to that of the thermolysin substrate Z-Gly-L-Leu-NH_2, was synthesized and examined. This simple peptide hydroxamic acid was fairly stable in buffer solution and gave a K_i value of 13 μM, five times greater than that of Z-Agly-L-Leu-NHOH. The difference of K_i values would be attributable to an interaction at a secondary binding site of the enzyme which prefers an azaglycyl residue to a normal glycyl residue.

TABLE II: Inhibition of Thermolysin by Peptide Hydroxamic Acid Derivatives.[a]

Hydroxamic acid	K_i (μM)
L-Leu-NHOH	190
Z-L-Leu-NHOH	10
Z-Gly-L-Leu-NH_2	21 000[b]
Z-Gly-L-Leu-NHOH	13
Z-Gly-D-Leu-NHOH	59
Z-Gly-L-Leu-$N(CH_3)OH$	2230
Z-Gly-L-Leu-$NHOCH_3$	No Inhib
Z-Agly-L-Leu-NHOH	2.7
Z-Gly-Gly-NHOH	940
Z-Gly-Gly-L-Leu-NHOH	39
Z-Gly-Gly-D-Leu-NHOH	250
HONH-Bzm-OEt[c]	20
HONH-Bzm-L-Ala-Gly-NH_2[c]	0.66
HONH-Bzm-L-Ala-Gly-NA[c]	0.43[d]

[a] 0.1 M Tris-HCl (pH 7.2), 2% dimethylformamide, 25 °C. [b] K_M value measured at pH 7.0 (Morihara and Tsuzuki, 1970). [c] The benzylmalonyl (Bzm) residue is racemic: Bzm = -CO-$CH(CH_2C_6H_5)$CO-. [d] I_{50} value at substrate concentration, 1 mM.

In order to elucidate the mechanism of inhibition, a number of hydroxamic acids with various sequences covering substitutions at the hydroxamic acid functional group, optical isomers, varying side chains, and peptide chain lengths were synthesized and examined for their inhibitory activity. The resulting K_i values which were determined by Dixon plots are summarized in Table II. The protection of the *N*-hydroxy group of the hydroxamic acid with a methyl group resulted in complete loss of the inhibitory activity. Even the substitution with methyl at nitrogen caused a 170-fold increase of the K_i value. The results suggest that the hydroxamic acid functional group is essential and probably chelates with the zinc atom in the enzyme–inhibitor complex. From the comparison of K_i values of the optical isomers of di- and tripeptide hydroxamic acids, the preference of a L-leucyl residue to a D isomer is obvious. However, the difference of K_i values by five to six times is too little to postulate distinct stereospecificity. In contrast, side-chain specificity is much more important, since the K_i value of Z-Gly-L-Leu-NHOH is 72 times smaller than that of Z-Gly-Gly-NHOH. This fact indicates that a hydrophobic side chain adjacent to the hydroxamic acid functional group is important for binding. Even the D isomer was a 16-fold better inhibitor than the nonspecific hydroxamic acid (Z-Gly-Gly-

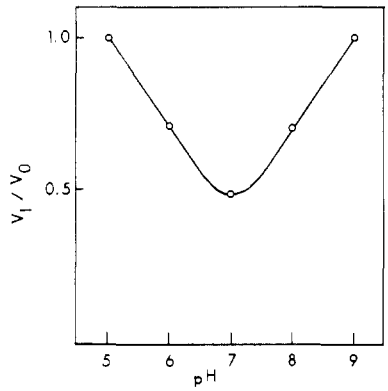

FIGURE 1: Plot of v_I/v_0 as a function of pH for the inhibitor Z-Gly-L-Leu-NHOH. v_0 is the rate of hydrolysis of furylacryloyl-Gly-L-Leu-NH_2 at 25 °C in buffers containing 2% dimethylformamide with a thermolysin concentration of 0.42 μM. v_I is the corresponding rate with the addition of 13 μM Z-Gly-L-Leu-NHOH. The buffers 0.1 M acetate (pH 5.0), 0.1 M citrate (pH 6.0), Tris-HCl (pH 7.0–9.0) were used.

NHOH). Peptide chain length had no significant effect as was the case with the hydrazides. The K_i values slightly increased from 10 to 39 μM with the elongation of the peptide chain from an amino acid to di- and tripeptide hydroxamic acids. In the series of D isomers, similar increases in K_i values were observed.

Benzylmalonyl Derivatives. Since there remained uncertainty in the binding mode in the series of Z-Gly_n-D, or L-Leu-NHOH (n = 1 or 2), we designed a new type of hydroxamic acid, benzylmalonyl derivatives which were expected to bind at the S_1' subsite[2] with the benzyl group and at the zinc atom with the hydroxamic acid functional group. Ethyl 2-(*N*-hydroxycarboxamido)-3-phenylpropanoate (HONH-Bzm-OEt) gave a K_i value of 20 μM. Considerable enhancement in the inhibition was achieved by attachment of a dipeptide to the inhibitor, and the K_i value decreased by 30 times. This suggests that significant interaction exists between HONH-Bzm-L-Ala-Gly-NH_2 and the extended substrate-binding region of thermolysin. The *p*-nitroanilide appeared to be the best inhibitor in this series, but a K_i value was not determined because of its nonlinear behavior in a Henderson plot. The I_{50} value, which is the inhibitor concentration necessary to depress the enzyme activity to 50%, is given in Table II.

The pH dependence of the inhibition by the hydroxamic acid inhibitor Z-Gly-L-Leu-NHOH was examined at several points around pH 7.2 which is the optimum pH for thermolysin hydrolysis of furylacryloyl-Gly-L-Leu-NH_2 and other substrates (Feder and Schuck, 1970) and appeared to have a bell-shaped curve with a minimum at pH 7.0 (Figure 1). The effect of the calcium chloride concentration was also tested up to 10 mM, but no difference in the inhibition was observed. This fact eliminates the possibility that the hydroxamic acid inhibitor coordinates with calcium which is required to stabilize the thermolysin structure.

Several inhibitors (Z-Gly_n-D, L-Leu-NHOH, n = 1 or 2) were checked for hydrolysis by thermolysin. Incubation of 10 mM of inhibitors with the L configuration with 20 μM of thermolysin in 0.1 M Tris-HCl buffer (pH 7.2) at 30 °C resulted in cleavage to yield L-Leu-NHOH which was detected by TLC on silica gel G using 1-butanol–acetic acid–pyridine–water (4:1:1:2) as solvent. No cleavage was observed for D isomers. The thermolysin concentration in this experiment was approximately 50 times higher that used in the inhibition experiment. Even so, hydrolysis was so slow (half life = ~48 h for both L isomers) that it would have had no effect on the inhibition reactions.

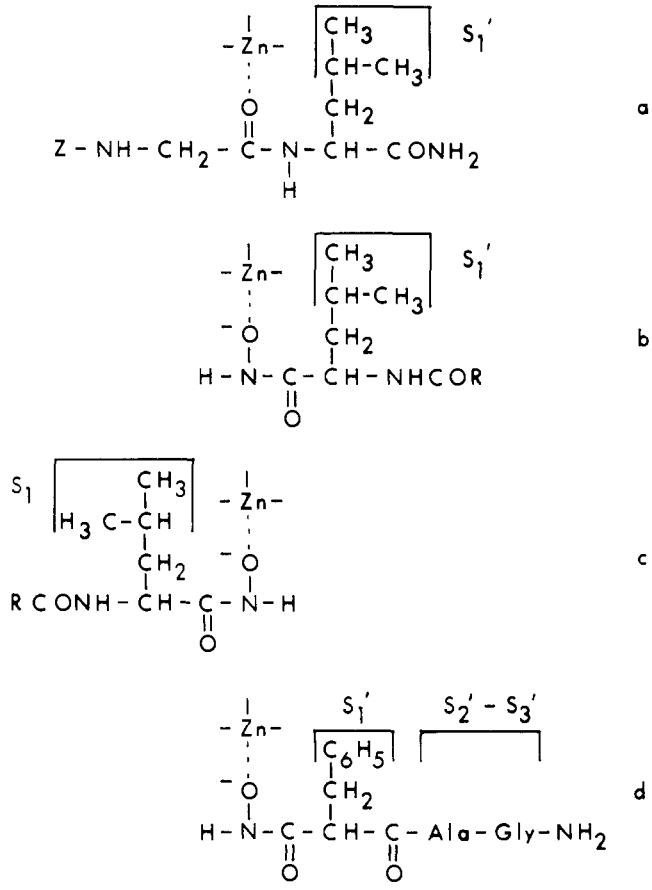

FIGURE 2: Schematic drawings showing the binding of a substrate (a) to the active site of thermolysin and possible binding modes for hydroxamic acid inhibitors (b–d).

Discussion

Electron-density maps of thermolysin and its complexes with peptides have provided a detailed description of the molecular structure of this enzyme and its mode of binding substrates (Kester and Matthews, 1977). In an enzyme–substrate complex, the carbonyl oxygen of the scissile peptide bond is coordinated to the zinc atom of the enzyme (Figure 2a). The primary specificity of thermolysin is determined by the interaction of the side chain of the P_1' amino acid residue with the hydrophobic S_1' pocket[2] formed from the side chains of Leu-202, Val-139, and other nonpolar groups. Particularly favorable residues are Ile, Leu, Phe, and Val (Morihara et al., 1968; Morihara and Tsuzuki, 1970; Feder and Schuck, 1970). The S_1 subsite, composed primarily of the side chain of Phe-114, is also hydrophobic. Preferred residues at this subsite are the hydrophobic residues Trp and Tyr along with polar residues such as Arg, Asn, Ser, Thr, and Gln (D. V. Meyers, A. D. Harley, and J. C. Powers, unpublished observations). The discussion of the binding mode of peptide hydroxamic acids will be based on this model.

Several dipeptide derivatives which contain hydrophobic amino acids are known competitive inhibitors of thermolysin (Feder et al., 1974). The dissociation constants of the inhibitors with thermolysin are all in the millimolar range, the K_i values of Z-L-Leu-Gly-NH_2 and Z-Gly-L-Leu-OH are, respectively, 3.07 and 5.65 mM. The K_M value for the thermolysin hydrolysis of Z-Gly-L-Leu-NH_2 is even higher, being 21 mM at pH 7.0. The hydroxamic acid functional group intensifies the binding. Replacement of the NH_2 group in Z-Gly-L-Leu-NH_2 with NHOH results in a 1600-fold tighter binding to thermolysin.

The fact that hydroxamic acids are bound so much more tightly to thermolysin than hydrazides or the corresponding peptides suggests that the hydroxamic acid functional group is coordinating the zinc atom in the active site of thermolysin. Consistent with this is the observation that replacement of the *N*-hydroxy group with a *N*-methoxy group resulted in loss of inhibitory activity. The *N*-methyl derivative Z-Gly-L-Leu-$N(CH_3)$OH is a 170 times poorer inhibitor than Z-Gly-L-Leu-NHOH, but is still bound nine times more tightly than Z-Gly-L-Leu-NH_2. The methyl group could either be causing steric hindrance between the inhibitor and some portion of the enzyme or may be electronically affecting the strength of the interaction between the zinc and the hydroxamic acid functional group.

Since hydroxamic acids are good bidentate ligands for metals in solution, the possibility that the inhibitors are selectively competing with thermolysin for either zinc or calcium ions should also be considered. Both possibilities can be eliminated. Added calcium has no effect on the inhibition of thermolysin by Z-Gly-L-Leu-NHOH, and thermolysin can be eluted unchanged from an agarose column with an attached HONH-Bzm-L-Ala-Gly- ligand (N. Nishino and J. C. Powers, unpublished observations), thus showing that the inhibitors are not removing zinc from the enzyme.

Both the hydroxamic acid functional group and an amino acid residue with a hydrophobic side chain are important for tight binding of the inhibitors to thermolysin. Z-Gly-Gly-NHOH is a 72-fold poorer inhibitor than Z-Gly-L-Leu-NHOH. In the series, Z-L-Leu-NHOH, Z-Gly-L-Leu-NHOH, and Z-Gly-Gly-L-Leu-NHOH, there is no significant decrease of K_i values with elongation of the peptide chain; in fact, there is a slight increase. Morihara and Tsuzuki (1970, 1971) have reported differences in the binding efficiency of oligopeptide substrates, which indicate that thermolysin possesses at least four subsites. Interactions at three subsites (S_1, S_1' and S_2') with peptides or peptide-like inhibitors have been observed crystallographically (Kester and Matthews, 1977; Weaver et al., 1977). If the acyl group of the various acylleucinehydroxamic acids were interacting significantly with the extended substrate-binding region of thermolysin, substantial differences in K_i values should have been observed. The possibility that all the inhibitors were being hydrolyzed to L-Leu-NHOH was considered and eliminated. First, L-Leu-NHOH is a much poorer inhibitor than the acylleucinehydroxamic acids, and, even though Z-Gly-L-Leu-NHOH and Z-Gly-Gly-L-Leu-NHOH are hydrolyzed in a concentrated thermolysin solution to L-Leu-NHOH, the rate is so slow ($t_{1/2}$ = ~48 h) that the hydrolysis would have no effect on the inhibition of thermolysin.

Two models can be postulated for the binding of acylleucinehydroxamic acid to thermolysin. In the first, the inhibitor is oriented in the inverse direction in the active site of thermolysin with the leucyl side chain of the inhibitor in the S_1' subsite and the hydroxamic acid functional group coordinated to the active-site zinc atom (Figure 2b). In the second model, the inhibitor is oriented in the normal direction with the leucyl side chain interacting with the S_1 subsite and again with the hydroxamic acid coordinating to zinc (Figure 2c). In order to differentiate between the two models, the optical antipodes Z-Gly-D-Leu-NHOH and Z-Gly-Gly-D-Leu-NHOH were examined. If the inverse model (Figure 2b) were correct, the D isomers should have been more effective inhibitors, since the acyl group could have interacted with the S′ subsites and the zinc atom, respectively. However, since in both cases the D isomers were four- to sixfold poorer inhibitors than the L isomers, this model appeared less probable than the "normal" model illustrated in Figure 2c. Nevertheless, recent crystallographic studies have shown that Z-Gly-Gly-L-Leu-NHOH binds at the S′ subsites (Brian Matthews and Margaret Holmes, personal communication).

Since there seemed to be no way to enhance the inhibitor activity of substrate analogues such as Z-Gly-L-Leu-NHOH by means of interactions with thermolysin's extended substrate-binding site, we designed the benzylmalonyl derivatives. Here the results clearly favor interaction with thermolysin's extended substrate-binding region and the model illustrated in Figure 2d. The racemic benzylmalonylhydroxamic acid HONH-Bzm-OEt is almost as effective an inhibitor as Z-Gly-L-Leu-NHOH. Addition of a peptide chain gives the most effective thermolysin inhibitors which we have observed and an over 30-fold drop in the K_i value. The S_2' subsite of thermolysin is a hydrophobic region near Phe-130 and Leu-202 with Asn-112 which is able to hydrogen bond to the peptide backbone of a P_2' residue (Weaver et al., 1977). An Ala residue at P_2' in a substrate is a slightly favored, while a Gly at P_3' is neutral (D. V. Meyers, A. D. Harley, and J. C. Powers, unpublished observations). The decrease in the K_i value upon attachment of a dipeptide at the C terminal of the P_1' benzylmalonylhydroxamic acid indicates that there exists at least a favorable interaction between the S_2' subsite and the P_2' residue of the inhibitor.

It should be noted that it is possible that the hydroxamic acid functional group in Figure 2 is acting as a bidentate ligand, since some five coordinate zinc complexes have been observed. Although X-ray studies have demonstrated that the hydroxamic acid functional group of two of the inhibitors is interacting with the zinc atom of thermolysin, the exact structure around the zinc is still unclear (Brian Matthews and Margaret Holmes, personal communication).

The specificity of one of the better thermolysin inhibitors was examined with a number of related enzymes. No inhibition of chymotrypsin A_α, leucine aminopeptidase, carboxypeptidase A, or *Clostridium histolyticum* collagenase by HONH-Bzm-L-Ala-Gly-NH_2 was observed. Chymotrypsin is a serine protease which recognizes aromatic side chains. The other three enzymes are all zinc metalloproteases related to thermolysin but with different substrate specificities. These results support the conclusion that the peptide hydroxamic acids are not general metal-coordinating agents but are recognizing both the active zinc and the substrate-recognition site of thermolysin.

In conclusion, a potent and specific series of reversible inhibitors for the metalloprotease thermolysin has been developed. The inhibitors have already proved useful as ligands for affinity chromatography of thermolysin and the *B. subtillis* neutral proteases A and B (N. Nishino and J. C. Powers, unpublished observations). A model for their interaction with the enzyme has been proposed based on the mode of interaction of peptide substrates with and the mechanism of catalysis by thermolysin. Using this model as a working hypothesis, work is currently underway directed toward the synthesis of inhibitors for other metalloproteases, including biologically important enzymes such as collagenase. In addition, crystallographic studies in the laboratory of B. W. Matthews at the University of Oregon should shortly produce more information upon the mode of binding of these inhibitors to thermolysin.

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Supplementary Material Available

Experimental details for the synthesis of the new compounds reported (12 pages). Ordering information is given on any current masthead page.

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Cobalt Exchange in Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: The preparation of metal hybrid species of horse liver alcohol dehydrogenase is made possible by the development of carefully delineated systems of metal ⇆ metal exchange employing equilibrium dialysis. The conditions which are optimal for the site-specific replacement of the catalytic and/or noncatalytic zinc atoms of the native enzyme by cobalt are not identical with those which are utilized for substitution with ^{65}Zn. Thus, while certain ^{65}Zn hybrids can be prepared by exploiting the differential effects of buffer anions, the cobalt hybrids are generated by critical adjustments in the pH of the dialysate. Factors which may determine the mechanism of metal replacement reactions include acid-assisted, ligand-assisted, and metal-assisted dechelation, steric restriction, and ligand denticity as well as physicochemical properties of the enzyme itself. The spectral characteristics of the catalytic and noncatalytic cobalt atoms reflect both the geometry of the coordination complexes and the nature of the ligands and serve as sensitive probes of these loci in the enzyme.

The role(s) of the intrinsic metal atom(s) of LADH in its catalytic mechanism are presently unknown. Thus far, studies of the kinetics and reaction mechanisms of transition metal model complexes have yielded little information pertaining to the manner in which their chemical properties relate to those of the enzyme·metal coordination complex or to the interactions between the metal atom and the ligands of the enzyme.

We have recently identified the catalytic (C) and noncatalytic (N) metal atoms of horse liver alcohol dehydrogenase [$(LADH)Zn_2Zn_2$] utilizing the differential properties of cobalt-substituted derivatives.[1] Such identification requires the preparation of a series of hybrid enzymes in which the extent and site-specificity of metal replacement are delineated precisely (Sytkowski & Vallee, 1975, 1976). The chemical properties characteristic of the respective metal binding sites become apparent as a consequence.

The present study describes the characteristics of metal exchange in LADH which constitute the basis of the preparation of these hybrid enzymes. Importantly, the conditions

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[1] Abbreviations used: $[(LADH)Zn_2Zn_2]$ or LADH, native horse liver alcohol dehydrogenase; CD, circular dichroism; EPR, electron paramagnetic resonance. In order to differentiate and clarify presentation, the first pair of exchangeable metal (M) atoms in the standard formulation is designated the "N" (noncatalytic) and the second the "C" (catalytic) pair, i.e., $[(LADH)N_2C_2]$. Hence, Zn and Co represent the (N) pair in $[(LADH)Zn_2M_2]$ and $[(LADH)Co_2M_2]$, while they are the (C) pair in $[(LADH)M_2Zn_2]$ and $[(LADH)M_2Co_2]$.

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