Design of Potent Reversible Inhibitors for Thermolysin. Peptides Containing Zinc Coordinating Ligands and Their Use in Affinity Chromatography†

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ABSTRACT: A series of potent inhibitors for the zinc metalloprotease thermolysin have been developed. Most contain the same tripeptide backbone (-Phe-L-Ala-Gly-NH_2) to interact with thermolysin's extended substrate-binding site and a ligand to coordinate to the zinc atom in the active site of the enzyme. The most effective inhibitors with K_I values in the 10^{-6}–10^{-7} M range at pH 7.2 and 25 °C were those with a hydroxamic acid, thiol, phosphoramidate, or *N*-hydroxy peptide functional group to coordinate to the zinc atom. Most of the inhibitors act as monodentate ligands for the zinc, and their effectiveness is determined by a combination of their ligating ability and their resemblance to the tetrahedral transition state for peptide bond hydrolysis. The *N*-hydroxy peptide and hydroxamic acids are proposed to act as bidentate ligands with both oxygen atoms interacting with the zinc atom. Hydroxamic acids were shown to exhibit the expected side-chain specificity. The derivative with a leucyl side chain at P_1' was a slightly better inhibitor (K_I = 0.48 μM) than that with a phenylalanyl side chain (K_I = 0.66 μM). The hydroxamic acid with no side chain was an extremely poor inhibitor (K_I = 1100 μM). A model was proposed to account for the inhibition of several aminopeptidases (metalloexopeptidases) by the natural inhibitors bestatin and amastatin. Two oxygen atoms of the α-hydroxycarbonyl functional group of the inhibitors are proposed to act as a bidentate ligand for the active-site zinc in a manner similar to that proposed for the *N*-hydroxy peptides and hydroxamic acids. One of the best hydroxamic acid inhibitors was attached to agarose via an aminopropyl spacer group and was shown to be a very potent and stable affinity support. Thermolysin was retained by the column and could be eluted under a variety of conditions. As expected, chymotrypsin and carboxypeptidase A were not retained. Two neutral proteases from *Bacillus subtilis* which have a substrate specificity similar to that of thermolysin could be separated on the column by gradient elution. The results reported should be useful for the design of specific inhibitors or affinity supports for other zinc metalloproteases.

Metalloproteases are widely distributed in nature and are involved in many important physiological processes including digestion (carboxypeptidase A and B), regulation of blood pressure (angiotensin-converting enzyme), and tissue remodeling and turnover (collagenase). Although there are considerable differences between various metalloproteases in terms of their substrate specificity, they all possess an essential zinc atom in their active sites and have similar catalytic mechanisms. Much of our knowledge of metalloprotease structure and function is based on studies with more accessible members of the family such as thermolysin and carboxypeptidase A and B. The three-dimensional structures of these three enzymes have been determined by X-ray crystallography (Matthews et al., 1974; Hartsuck & Lipscomb, 1971; Schmid & Herriott, 1976).

Several types of natural and synthetic low molecular weight reversible inhibitors of metalloproteases are known. The potent thermolysin inhibitor phosphoramidon (Komiyama et al., 1975b) and the aminopeptidase inhibitors bestatin (Suda et al., 1976; Nishizawa et al., 1977) and amastatin (Aoyagi et al., 1978) have been isolated from cultural filtrates of actinomycetes. Benzylsuccinic acid (Byers & Wolfenden, 1972, 1973; Zisapel & Sokolovsky, 1974) and its analogues (McKay & Plummer, 1978) have been described as "bi-product" inhibitors of carboxypeptidase A and B. A group at Squibb has developed a series of mercaptoalkanoyl amino acids such as $HSCH_2CH(CH_3)$CO-L-Pro-OH as inhibitors of the angiotensin-converting enzyme and are testing them clinically for the treatment of hypertension (Cushman et al., 1977). Recently, we have shown that peptide hydroxamic acids (Nishino & Powers, 1978) and phosphoryl peptides (Kam et al., 1979) are potent inhibitors of thermolysin.

Although there is considerable variety in the structures of the various inhibitors, as might be expected based on the differing substrate specificity of the enzymes studied, all the inhibitors contain a functional group which can act as a ligand for the zinc atom in the active sites of the various metalloproteases. X-ray crystallographic studies of thermolysin complexed with phosphoramidon (Kester & Matthews, 1977) or benzylsuccinic acid (Bolognesi & Matthews, 1979) have shown that these inhibitors bind to the enzyme with an oxygen atom of the phosphoryl group or the carboxyl group acting as a monodentate ligand for the active-site zinc atom. Similar studies with peptide hydroxamic acids indicate that the hydroxamic acid functional group is not acting as a simple monodentate ligand (B. Matthews and M. Holmes, private communication). Indeed, bestatin has been proposed to be acting as a bidentate ligand for the active-site zinc atom of aminopeptidase B and leucine aminopeptidase (Nishizawa et al., 1977).

In order to evaluate the effectiveness of the various types of inhibitors which have been reported for metalloproteases, we have chosen to study thermolysin and one peptide backbone to which the various coordinating ligands were attached. In addition to functional groups based on the metalloprotease inhibitors previously reported, we have investigated other functional groups including some unique *N*-hydroxy peptides which could bind as either bidentate or monodentate ligands. In this paper we report the results of these studies and the

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attachment of one of the ligands to an affinity resin and its use in the affinity purification of thermolysin and two *Bacillus subtilis* neutral proteases.

Materials and Methods

Thermolysin was purchased from the Sigma Chemical Co. Furylacryloyl-Gly-L-Leu-NH_2 was purchased from Vega–Fox Biochemicals. Diethyl isobutylmalonate, diethyl benzylmalonate, *N,N'*-dicyclohexylcarbodiimide, *N*-hydroxybenzotriazole, and *O*-benzylhydroxylamine hydrochloride were the products of the Aldrich Chemical Co., Inc. *N*-Hydroxysuccinimide was obtained from the Eastman Kodak Co. Affi-Gel 101 (aminopropylagarose) was purchased from Bio-Rad Laboratories. The syntheses of all new compounds except those used in the preparation of the affinity adsorbent are described in the supplementary material (see supplementary material paragraph at end of paper).

2-(Ethyloxycarbonyl)-3-phenylpropanoyl-L-alanylglycine Benzyl Ester (EtOCOCH($CH_2C_6H_5$)CO-L-Ala-Gly-OBzl).[1] To a chilled solution of monoethyl benzylmalonate dicyclohexylamine salt (1.62 g, 4 mmol) and H-L-Ala-Gly-OBzl·HCl (1.09 g, 4 mmol) in CH_2Cl_2 (20 mL) was added *N,N'*-dicyclohexylcarbodiimide (0.824 g, 4 mmol). The reaction mixture was stirred overnight at room temperature. After the *N,N'*-dicyclohexylurea was removed by filtration, the filtrate was washed successively with 2% HCl, 4% $NaHCO_3$, and water. The solution, after being dried with $MgSO_4$, was concentrated, and the product was crystallized from CH_2Cl_2–petroleum ether. Recrystallization from ethanol–petroleum ether gave 1.41 g (80%), mp 91–92 °C.

Anal. Calcd for $C_{24}H_{28}N_2O_6$: C, 65.44; H, 6.41; N, 6.36. Found: C, 65.36; H, 6.43; N, 6.34. The corresponding acid was prepared by hydrogenolysis.

2-(Ethoxycarbonyl)-3-phenylpropanoyl-L-alanylglycine–Affi-Gel 101. Affi-Gel 101 (50 mL, 0.38 mmol of amino groups), obtained as a suspension in water, was washed with gradually increasing concentrations of dimethylformamide in water until the final suspension was in dry dimethylformamide (Hjerten et al., 1974). The active ester of EtOCOCH($CH_2C_6H_5$)CO-Ala-Gly-OH (665 mg, 1.9 mmol) was prepared from *N,N'*-dicyclohexylcarbodiimide (433 mg, 2.1 mmol) and *N*-hydroxysuccinimide (242 mg, 2.1 mmol) in dimethylformamide (10 mL) by reaction for 3 h at room temperature. The filtrate, after removal of dicyclohexylurea, was added to the suspension of Affi-Gel 101, and the mixture was gently stirred for 15 h. Additional *N,N'*-dicyclohexylcarbodiimide (206 mg, 1 mmol) was added, and stirring was continued for 24 h. Acetic anhydride (1 mL, 10 mmol) was added to block any remaining unreacted amino groups. After 3 h the suspension was placed on a glass filter and washed thoroughly with dry dimethylformamide. The gel gave a negative reaction in the ninhydrin test (Kaiser et al., 1970) for free amino groups. The acid hydrolysate of 1 mL of settled gel in water gave 10 μmol each of alanine and glycine. The starting resin contained 7.6 μmol of amino groups/mL. The higher apparent incorporation of 10 μmol of peptide/mL is attributed to the fact that the resin shrunk during the reaction due to the dimethylformamide, and therefore the content of amino groups per milliliter of the resin was higher than expected.

2-(N-Hydroxycarboxamido)-3-phenylpropanoyl-L-alanylglycine–Affi-Gel 101. To a solution of NH_2OH·HCl (2.78 g, 40 mmol) in dimethylformamide (20 mL) was added NEt_3 (5.6 mL, 40 mmol), and the precipitated NEt_3·HCl was removed by filtration. A mixture of EtOCOCH($CH_2C_6H_5$)-CO-Ala-Gly–Affi-Gel 101 and *N*-hydroxybenzotriazole (135 mg, 1 mmol) as catalyst (Burton & Haber, 1975) was gently stirred for 5 days in the NH_2OH–dimethylformamide solution. The gel was thoroughly washed as described above. Then the solvent was gradually exchanged with water.

Enzymatic Assay. Furylacryloyl-Gly-L-Leu-NH_2 was employed to measure the thermolysin and neutral protease activities (Feder, 1968). The decrease of absorbance at 345 nm was followed with a Beckman Model 25 spectrophotometer using 2.0 mL of substrate solution (1 mM) containing 0.1 M Tris-HCl and 1 or 2% dimethylformamide at 25 °C and pH 7.2.

For determination of K_I values of the inhibitors by Dixon plots, we employed 1 and 2 mM substrate concentrations. Degassed buffer was used in the case of the thiol compound.

Affinity Chromatography of Thermolysin. The affinity adsorbent was packed in a column (0.9 × 10 cm). The column was first equilibrated with 0.1 M Tris-HCl and 0.01 M $CaCl_2$, pH 7.2. The enzyme (4 mg) was applied in 8 mL of this buffer, and the column was washed with 20 mL of buffer. Fractions of 2.0 mL were collected. The bound protein was then eluted with 0.1 M Tris-HCl and 0.1 M $CaCl_2$, pH 9.0, with a flow rate of 20 mL/h.

Affinity Chromatography of Neutral Proteases A and B of B. subtilis. The column (0.9 × 10 cm) was equilibrated with 0.1 M Tris-HCl and 0.01 M $CaCl_2$, pH 7.2. Crude enzyme (40 mg) was applied in 2 mL of this buffer. After thorough washing (20 mL) the enzymes were eluted separately by a linear gradient using 0.1 M Tris-HCl and 0.05 M $CaCl_2$, pH 8.0 (20 mL), to 0.1 M Tris-HCl and 0.1 M $CaCl_2$, pH 9.0 (20 mL). Fractions of 2.0 mL were collected at a flow rate of 20 mL/h.

Results

In order to test the effectiveness of the various ligands, we felt it was essential to have the same peptide backbone structure in each inhibitor. The primary substrate recognition subsite (S_1')[2] of thermolysin prefers amino acid residues with aromatic or hydrophobic side chains such as those from Leu or Phe residues. The S_2' subsite prefers Ala and several other amino acid residues (D. V. Myers, A. D. Harley, and J. C. Powers, unpublished experiments). Whether a P_3' residue is significant is as yet unclear. Therefore, all the inhibitors were designed to contain the CH($CH_2C_6H_5$)CO-L-Ala-Gly-NH_2 or CH[$CH_2CH(CH_3)_2$]CO-L-Ala-Gly-NH_2 to interact with the S_1'–S_3' subsites of thermolysin in the same manner as a substrate with the sequence -Phe-Ala-Gly- or -Leu-Ala-Gly-. The various ligating groups were then attached in the region which would be occupied by the scissile peptide bond in a substrate.

The inhibitors studied and their K_I values are reported in Table I. The K_I values were determined by means of Dixon plots using two separate concentrations of the substrate (furylacryloyl-Gly-L-Leu-NH_2). At each substrate concentration, rates were determined with five different inhibitor concentrations. Under the conditions of the assay ($K_M \gg$ [S]), it is not possible to distinguish between competitive and noncompetitive inhibition. However, we have recently synthesized a new fluorogenic substrate for thermolysin and used

[1] Abbreviations used: Bzm, benzylmalonyl [–COCH($CH_2C_6H_5$)CO–]; Ibm, isobutylmalonyl [–COCH($CH_2CH(CH_3)_2$CO–]; Mal, malonyl; Bzl, benzyl; Et, ethyl; Boc, *tert*-butyloxycarbonyl; Ac, acetyl; CHO, formyl; HOLeu, *N*-hydroxyleucine; Z, benzyloxycarbonyl.

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

Table I: K_I Values of Inhibitors of Thermolysin[a]

inhibitors	K_i (μM)
HONH-Bzm-L-Ala-Gly-NH_2[b]	0.66
HONH-Bzm-L-Ala-Gly-OH	0.65
HONH-Ibm-L-Ala-Gly-NH_2	0.48
HONH-Mal-L-Ala-Gly-NH_2	1100
CHO-HOLeu-L-Ala-Gly-NH_2	3.8
Ac-HOLeu-L-Ala-Gly-NH_2	3400
HO-Bzm-L-Ala-Gly-NH_2	420
$HSCH_2CH(CH_2C_6H_5)CO$-L-Ala-Gly-NH_2	0.75
$(^-O)_2(O{=})$P-L-Phe-L-Ala-Gly-NH_2	2.6
$^-O(S^-)(O{=})$P-L-Phe-L-Ala-Gly-NH_2	7.0
$^-O(O{=})_2$S-L-Phe-L-Ala-Gly-NH_2	4300
CH_3SO_2-L-Phe-L-Ala-Gly-NH_2	NI[c]
$HOCH_2CO$-L-Phe-L-Ala-Gly-NH_2	2900
CH_3COCH_2CO-L-Phe-L-Ala-Gly-NH_2	3100

[a] 0.1 M Tris-HCl, pH 7.2, and 1% dimethylformamide, 25 °C. [b] Data from Nishino & Powers (1978). [c] NI = no inhibition.

it to show that NONH-Bzm-Ala-Gly-NH_2 is purely a competitive inhibitor of thermolysin (N. Nishino and J. C. Powers, unpublished experiments).

Affinity Chromatography of Thermolysin and B. subtilis Neutral Proteases. The utility of the peptide hydroxamic acid inhibitor for chromatographic purification of metalloproteases was investigated in order to demonstrate the value of these types of compounds for future studies with other important metalloproteases. The adsorbent was prepared by coupling of $EtOCOCH(CH_2C_6H_5)CO$-L-Ala-Gly-OH to aminopropylagarose in dimethylformamide using the active ester method. Prior removal of the water from the adsorbent by successive treatment with dimethylformamide allowed this reaction to be carried out in nonaqueous medium. Although analysis for amino groups indicated essentially complete reaction of the peptide with agarose, the agarose was treated with acetic anhydride to block any remaining amino groups and prevent any ion-exchange effects. The hydroxamic acid was then prepared by reaction of the ester with NH_2OH, using *N*-hydroxybenzotriazole to catalyze the reaction. The hydroxamic acid functional group was introducted at the last step since its presence during coupling would have resulted in other types of reaction. To our knowledge this is the first example of a hydroxamic acid which has been attached to an affinity resin. The synthetic procedure is general and should be equally applicable to peptide hydroxamic acids with other amino acid sequences.

Thermolysin (Figure 1) and the neutral proteases from *B. subtilis* (Figure 2) were retained by the hydroxamic acid containing adsorbent at pH 7.2. The serine protease chymotrypsin and the zinc metalloexopeptidase carboxypeptidase A were washed through with the pH 7.2 buffer, indicating no affinity to the column. The precursor of the adsorbent, $EtOCOCH(CH_2C_6H_5)CO$-L-Ala-Gly–aminopropylagarose, did not bind any of the enzymes examined. Thus, the hydroxamic acid functional group is required for binding of thermolysin and *B. subtilis* neutral proteases.

Thermolysin was eluted from the column with 0.1 M Tris-HCl, pH 9.0, containing 0.1 M $CaCl_2$ (Figure 1). The high concentration of $CaCl_2$ was necessary for sharp emergence of the enzyme peak; at 0.01 M $CaCl_2$ the thermolysin did not elute in a reasonable period of time. The initial activity of the commercial sample of thermolysin was measured by using the furylacryloyl-Gly-L-Leu-NH_2 assay containing 2% dimethylformamide and was found to have a k_{cat}/K_M of 0.37 $(mg/mL)^{-1}$ s^{-1}. A small amount of inactive protein was washed through the column (Figure 1), and thermolysin, which was subsequently eluted, had a much higher k_{cat}/K_M value of

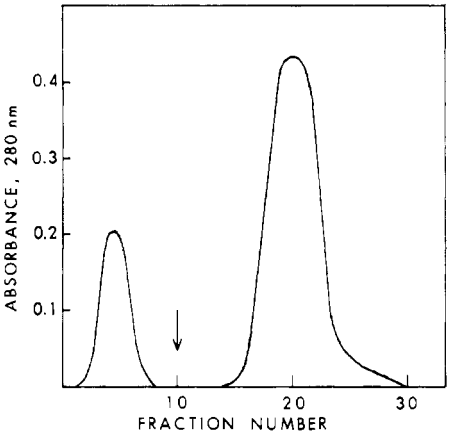

FIGURE 1: Affinity chromatography of thermolysin. The column (0.9 × 10 cm) was first equilibrated with 0.1 M Tris-HCl and 0.01 M $CaCl_2$, pH 7.2. The enzyme (4 mg) was applied in 8 mL of this buffer, and the column was washed with 20 mL of buffer. The bound protein was then eluted with 0.1 M Tris-HCl and 0.1 M $CaCl_2$, pH 9.0 (arrow), with a flow rate of 20 mL/h.

0.48 $(mg/mL)^{-1}$ s^{-1} (16×10^3 M^{-1} s^{-1}). In order to confirm that the inactive protein was not being formed on the column, we adjusted the eluant with the pH 9.0 buffer to pH 7.2 with acetic acid and reapplied it to the column. No protein washed through the column with pH 7.2 buffer, and a quantitative recovery of thermolysin with unchanged specific activity was obtained upon elution with the pH 9.0 buffer.

Other methods of eluting thermolysin were investigated both to confirm the mode of binding of the enzyme to the column and since we wished to have available a variety of conditions which would be used with other metalloproteases. High concentrations of NaCl (3 M) or guanidine hydrochloride (4 M) in 0.1 M Tris-HCl at pH 7.2 did not elute the enzyme from the column. This indicates that the binding mode was not simply a hydrophobic effect. 1,10-Phenanthroline (1 mM) in pH 7.2 buffer did elute the protein. This metal-chelating reagent also completely prevented the retention of thermolysin by the column when added to the enzyme solution before it was placed on the column. These facts indicate that apothermolysin (Latt et al., 1969) is not bound to the column, and coordination between the active-site zinc atom of thermolysin and the hydroxamic acid functional group of the ligand is primarily responsible for the retention of the active enzyme. This mode of elution could be utilized with any zinc metalloprotease that could easily be regenerated from the apoenzyme by the addition of zinc following chromatography. Zinc ion (1 mM), an inhibitor of thermolysin and many other metalloproteases, also effected a slow elution of the thermolysin.

Neutral proteases A and B of *B. subtilis* were eluted separately from the hydroxamic acid column by using a linear gradient of 0.1 M Tris-HCl and 0.05 M $CaCl_2$, pH 8.0, to 0.1 M Tris-HCl and 0.1 M $CaCl_2$, pH 9.0, as shown in Figure 2. A substantial quantity of inactive protein present in the crude enzyme preparation was removed before initiation of the gradient. Thermolysin was not eluted by this gradient, so conceivably a separation of all three enzymes could have been achieved. The k_{cat}/K_M values of the first and second peaks were respectively 0.12 and 0.36 $(mg/mL)^{-1}$ s^{-1} when assayed with furylacrylolyl-Gly-L-Leu-NH_2 in a buffer containing 2% dimethylformamide using the reported extinction coefficients of these enzymes (Pangburn et al., 1973).

The hydroxamic acid adsorbent has an extremely long lifetime and has been used in our laboratory for over 50 separate experiments with no noticeable degradation of the resin or change in efficiency.

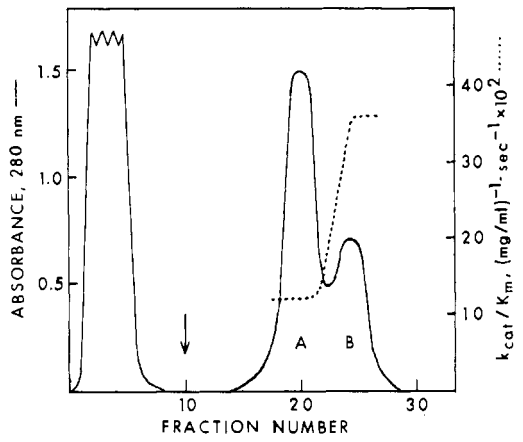

FIGURE 2: Affinity chromatography of neutral proteases A and B of *B. subtilis*. The column (0.9 × 10 cm) was equilibrated with 0.1 M Tris-HCl and 0.01 M $CaCl_2$, pH 7.2. Crude enzyme (40 mg) was applied in 2 mL of this buffer. After thorough washing (20 mL), the enzymes were eluted separately by a linear gradient (arrow) using 0.1 M Tris-HCl and 0.05 M $CaCl_2$, pH 8.0 (20 mL), to 0.1 M Tris-HCl and 0.1 M $CaCl_2$, pH 9.0 (20 mL). Fractions of 2.0 mL were collected at a flow rate of 20 mL/h.

Discussion

There is currently considerable interest in the development of specific inhibitors for metalloproteases since these enzymes have significant roles in important physiological processes such as fertilization (acrolysin) and in disease states such as arthritis (collagenase), bacterial infections (collagenase and elastase), and hypertension (angiotensin-converting enzyme). New metalloprotease inhibitors should be useful in the purification of metalloproteases and their characterization. Some may have therapeutic use. In fact, one such inhibitor (Squibb's captopril) is currently undergoing clinical testing in the treatment of hypertension.

Most natural and synthetic metalloprotease inhibitors which have been reported thus far have a peptide backbone which is recognized by the enzyme in question and a functional group which can act as a ligand for the active-site zinc atom. In this paper, we have attached all the various ligating groups to a single peptide backbone structure (Phe-L-Ala-Gly-NH_2) and have evaluated them with a single enzyme (thermolysin). Our goal was to discover which functional groups make good ligands for the zinc atom of metalloproteases, uncover information on their binding modes, and choose the functional groups which would be worthy of extending to other metalloproteases.

Monodentate Ligands. Several of the functional groups which we investigated can act only as monodentate ligands. These include the thiol, the phosphoramidate, the thiophosphoramidate, and the sulfamate. In fact, it appears that peptide substrates themselves could be considered to be monodentate ligands. The currently accepted mechanism of action of thermolysin (Kester & Matthews, 1977) involves binding of a substrate to the enzyme to form a complex (Figure 3a). A water molecule, which is the fourth zinc ligand in native thermolysin, is displaced by the oxygen atom of the scissile peptide bond. Subsequent bond hydrolysis takes place by the catalyzed addition of water to this peptide bond.

The thiol $HSCH_2CH(CH_2C_6H_5)$CO-L-Ala-Gly-NH_2 was one of the best inhibitors which we investigated (K_I of 0.75 μM). Both cysteine and β-mercaptoethanol were weak inhibitors ($K_I \simeq 1$ mM) of thermolysin. But combination of the thiol functional group with a peptide sequence which is recognized by thermolysin's extended substrate-binding region results in over a 1000-fold decrease in K_I. The proposed

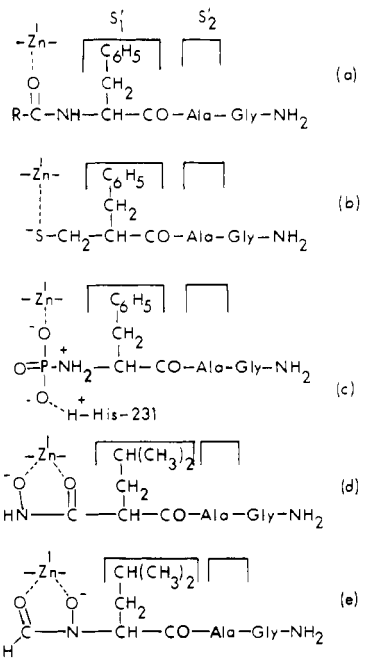

FIGURE 3: Schematic diagram showing the proposed interaction between the active site of thermolysin and (a) a substrate, (b) the mercapto inhibitor, (c) the phosphoramidate inhibitor, (d) the hydroxamic acid inhibitor, and (e) the *N*-hydroxy peptide inhibitor.

binding mode for the thiol is shown in Figure 3b. Due to the additional size of the sulfur atom and the conformational flexibility of the $HSCH_2$ group, there is little doubt that the thiol is effectively occupying the space occupied by the peptide bond of a substrate and is ligating the zinc atom. We synthesized the thiol following the report by Cushman et al. (1977) that 3-mercapto-2-methylpropanoyl-L-proline was an extremely potent inhibitor of the angiotensin-converting enzyme (K_I = 0.01 μM). A model similar to Figure 3b was proposed for that enzyme. It is clear from our results with thermolysin that not only do the two enzymes have closely related catalytic sites as distinguished from their substrate-binding sites but that the thiol functional group should be an extremely valuable group to incorporate into inhibitor structures for other metalloproteases. Indeed, Ondetti et al. (1979) have recently extended this class of inhibitor to carboxypeptidase A and B.

The phosphoramidate functional group is found in the natural thermolysin inhibitor phosphoramidon (Komiyama et al., 1975b). An X-ray crystallographic analysis of the thermolysin–phosphoramidon (rhamnose-P-Leu-Tyr-OH) complex has shown that one of the oxygens of the phosphoramidate functional group coordinates to the zinc atom of thermolysin (Weaver et al., 1977). The other oxygen is hydrogen bonded both to a water molecule and to the catalytically important Glu-143. The peptide carbonyl group of Ala-113 hydrogen bonds to the phosphoramidate NH. The phosphoramidate N is ~4.1 Å from His-231, too far for hydrogen bonding. Simple peptide phosphoramidates have been shown to be effective inhibitors of several metalloproteases (Kam et al., 1979). When a phosphoryl group was attached to the -Phe-Ala-Gly-NH_2 sequence, the resulting inhibitor was found to be an excellent thermolysin inhibitor (K_I = 2.6 μM), although slightly less effective than the thiol.

It is now widely accepted that enzymes stabilize the transition state for the catalyzed reaction. In the case of thermolysin, this would be a tetrahedral adduct of a water molecule to the scissile peptide bond in Figure 3a. The resemblance of the tetrahedral phosphoramidate functional group

to this transition state probably accounts for the tight binding of phosphoryl-L-Phe-L-Ala-Gly-NH_2 to thermolysin. Phosphoramidates with a P_1' leucyl residue will bind even more effectively and have K_I values 5–10-fold lower than those with a phenylalanyl residue.

The sulfamate $^-SO_3$-L-Phe-L-Ala-Gly-NH_2 also resembles the transition state for peptide bond hydrolysis and can act as a monodentate ligand for the zinc atom of thermolysin. However, it was a 1700-fold poorer inhibitor (K_I = 4.3 mM) than the phosphoramidate. Geometry or bond angle differences between the phosphoramidate and the sulfonamide can be eliminated from consideration since the bond lengths and angles for $^+NH_3PO_3^{2-}$ (P–N, 1.79 Å; P–O, 1.52 Å; ∠NPO, 103°; ∠OPO, 115°) are essentially identical with those for $^+NH_3SO_3^-$ (S–N, 1.76 Å; S–O, 1.44 Å; ∠NSO, 102°; ∠OSO, 116°) (Sutton, 1958). However, it does appear that the phosphoramidate functional group should be a better ligand than a sulfamic acid. Evidence for this can be found in comparing the pK value for NH_2SO_3H (pK = 1.0) with those for $NH_2PO_3H_2$ (pK_1 = 2.8, pK_2 = 8.2) and n-$BuNHPO_3H_2$ (pK_1 = 2.9, pK_2 = 9.9) (Benkovic & Sampson, 1971). The phosphoramidates hold onto the proton more tightly and thus have higher pK values (pK_1) than the sulfamic acid. They would be expected to behave similarly with another Lewis acid, the zinc atom in the active site of thermolysin.

Another reason for the tight binding of the phosphoramidate compared to the sulfamic acid could be interaction of the additional negatively charged oxygen of the phosphoramidate with some residue of thermolysin such as His-231. The monoanion of simple phosphoramidates such as n-$BuNHPO_3H_2$ has been shown to exist in a zwitterionic form similar to that depicted in Figure 3c (Benkovic & Sampson, 1971). An electrostatic interaction between the negatively charged phosphoramidate oxygen and the positively charged imidazole of His-231 either directly or through an intervening water molecule would be expected to stabilize the enzyme–inhibitor complex (Figure 3c). Support for this model was obtained by showing that thermolysin was inhibited 115-fold better by $(HO)_2PO$ amino acids as compared to (MeO)(HO)PO amino acids, the phosphoramidate monomethyl ester (Kam et al., 1979). Phosphoramidate monomethyl esters have approximately the same ligating ability as phosphoramidates as indicated by their respective pK values. The observed K_I difference must therefore be due to an additional interaction between thermolysin and phosphoramidates such as that shown in Figure 3c (Kam et al., 1979).

The thiophosphoramidate thiophosphoryl-L-Phe-L-Ala-Gly-NH_2 was synthesized to see if the best features of the phosphoramidate (resemblance to the transition state) and the thiol (sulfur ligating group) could be incorporated into one functional group. Its K_I value was 2.7-fold higher than that of the phosphoramidate. This could be attributed to the fact that a P–S bond (1.85 Å) is longer than a P–O bond (1.39 Å) (Sutton, 1958) and causes the thiophosphoryl group to be displaced slightly from the zinc atom of thermolysin. This could result in a decrease in some of the enzyme–inhibitor interactions such as the hydrogen bonding between the carbonyl group of Ala-113 and the thiophosphoramidate NH. Alternately, the thiophosphoryl peptide may be ligating to the zinc atom of thermolysin with one of its oxygen atoms instead of sulfur, and the difference in K_I values may be due simply to slight differences of electron density on the oxygens of the two functional groups.

Carbonic anhydrase is inhibited strongly by a wide variety of aromatic sulfonamides (Lindskog et al., 1971). X-ray crystallographic studies have shown that the sulfonamide functional group interacts directly with the zinc atom in the active site of the enzyme. The sulfonamide has replaced a water molecule as the fourth ligand around the zinc atom and the process is very reminiscent of the binding of a substrate to thermolysin. The sulfonamide CH_3SO_2-L-Phe-L-Ala-Gly-NH_2 was synthesized to test the possibility of such an interaction with thermolysin. No binding was observed. In the case of carbonic anhydrase the ionized form of the sulfonamide (RSO_2NH^-) has been shown to bind, indicating that the nitrogen atom is interacting with the zinc atom (Lindskog et al., 1971). Such an interaction is not likely with our tripeptide sulfonamide since the nitrogen atom would not be close to the zinc atom if it were bound to thermolysin's active site in a position similar to that occupied by a substrate.

Carboxylic acids have been shown to be inhibitors of four metalloproteases. Benzylsuccinic acid inhibits carboxypeptidase A and has been termed a "bi-product" analogue due to its resemblance to the products of peptide bond hydrolysis. Cushman et al. (1977) have shown that carboxyalkanoyl amino acids such as glutaryl-L-proline inhibit the angiotensin-converting enzyme. The potency of the inhibitors exhibited considerable variation as the distance between the carboxylate anion and the amino acid residue was varied. In an X-ray crystallographic study Bolognesi & Matthews (1979) have shown that benzylsuccinate binds to thermolysin (K_I = 3.8 mM) and acts as a monodentate ligand with the carboxylate which is β to the benzyl group coordinating to the zinc atom.

The benzylmalonyl dipeptide HO-Bzm-L-Ala-Gly-NH_2 is a better thermolysin inhibitor than benzylsuccinic acid. The ninefold difference reflects first the preference of thermolysin for inhibitors without a P_1' carboxylate and second the interaction of the peptide chain of HO-Bzm-L-Ala-Gly-NH_2 with thermolysin's extended substrate-binding region. The spacing betwen the α carbon of the P_1' residue and the scissile carbonyl oxygen of a peptide substrate (2.5 Å) is quite similar to that between the α carbon of the inhibitor and the carboxylate oxygen (2.3 Å). This spacing is longer in benzylsuccinic acid (3.2 Å), and the benzylmalonyl derivatives might be expected to have the optimum separation between the ligating oxygen and the α carbon of the P_1' residue. However, in the case of the angiotensin-converting enzyme, a glutaryl derivative of proline was a more effective inhibitor than either the succinyl or the malonyl derivative.

Comparison of the pK values of carboxylic acids and phosphoramidates indicates that the former should be equal or slightly better ligands. However, the phosphoramidate binds to thermolysin 160-fold better than the benzylmalonyl derivative. We attributed this difference to stabilization of the bound phosphoramidate by the enzyme due to its resemblance to the transition state for peptide hydrolysis and possibly due to an electrostatic interaction with His-231. In the case of the sulfamic acid which is also tetrahedral, its pK is 2 or 3 times lower than that for the carboxylic acid and it is a poorer ligand and thus bound 10-fold less tightly than the carboxylic acid even though the latter is not tetrahedral.

N-Hydroxy Peptides and Hydroxamic Acids. In a previous paper we reported that peptide hydroxamic acids were potent reversible inhibitors of thermolysin (Nishino & Powers, 1978). In the current series of inhibitors the (isobutylmalonyl)-hydroxamic acid HONH-Ibm-Ala-Gly-NH_2 had the lowest K_I and was slightly more potent than the thiol. The side-chain specificity of the hydroxamic acids was quite similar to that observed with substrates. The isobutylmalonyl derivative (K_I = 0.48 μM) bound slightly more tightly than the benzyl

derivative (K_I = 0.66 μM), as expected on the basis of thermolysin's preference for cleavage at leucyl residues over phenylalanyl residues in substrates. The importance of the S_1'–P_1' interaction in the hydroxamic acids is indicated by the fact that the unsubstituted malonyl derivative NONH-Mal-L-Ala-Gly-NH_2 was bound 2300-fold less tightly to the enzyme. The hydroxamic acid with a C-terminal carboxyl group in place of the carboxamide showed no change in K_I value, indicating that there is no significant interaction between the P_3' residue of the inhibitor and the enzyme. Since the substituted malonyl residues are racemic and the actual inhibitor is probably the one with the L configuration, the actual K_I of the correct isomer would be reduced by one-half.

The hydroxamic acid functional group could be interacting with thermolysin's active-site zinc atom either as a monodentate ligand or as a bidentate ligand as shown in Figure 3d. In solution hydroxamic acids coordinate to metals as bidentate ligands (Anderegg et al., 1963), and although no zinc–hydroxamic acid complexes have had their structures determined by X-ray crystallography, a number of pentacoordinate zinc complexes are known. Previous evidence for bidentate ligation is the 170- and >400-fold increases in K_I value observed respectively in *N*-methyl and *O*-methyl derivatives of a hydroxamic acid (Nishino & Powers, 1978).

In order to investigate further the state of ligation of the hydroxamic acid functional group, we synthesized the isomeric *N*-hydroxy peptide CHO-HOLeu-L-Ala-Gly-NH_2. If the hydroxamic acid were a bidentate ligand for thermolysin's zinc atom (Figure 3d), then the isomeric *N*-hydroxy peptide would be expected to interact in a similar fashion as shown in Figure 3e. Indeed, the *N*-hydroxy peptide was an excellent inhibitor with a K_I value of 3.8 μM, which was eightfold higher than that for the isomeric hydroxamic acid. If both inhibitors were acting as monodentate ligands, a much greater difference in K_I values would be expected since an amide carbonyl group is a much poorer metal ligand than the oxygen of a N–OH bond.

Substitution of an acetyl group for the formyl group in the *N*-hydroxy peptide structure (Figure 3e) resulted in a 900-fold increase in K_I. Part of this surprising difference is certainly due to p*K* differences. The p*K* of $CH_3CONHOH$ is ~0.6 p*K* units higher than the p*K* of CHONHOH due to the inductive effect of a methyl group compared to hydrogen (Abassi & Ahmed, 1976; Anderegg et al., 1963). Since the ionized form of the *N*-hydroxy peptide (or hydroxamic acid) is binding to the enzyme, the formyl derivative would be more dissociated at pH 7.2 and thus a better inhibitor. However, this cannot account totally for the 900-fold difference. One is forced to conclude that the substitution of a methyl for hydrogen sterically interferes with binding of the *N*-hydroxy peptide to the enzyme. A similar 170-fold decrease was observed when comparing hydroxamic acids with a HONHCO structure to the methyl derivative $HON(CH_3)CO$ (Nishino & Powers, 1978). Since a methyl group is electron releasing, both methyl derivatives (CH_3CONOH and CH_3NOHCO) would be expected to be better metal ligands; yet in both cases methyl derivatives were poorer inhibitors. If monodentate ligation was involved in each case, it is hard to rationalize a steric problem with the methyl derivatives since the methyl group would be in the region occupied by the α carbon of the P_1 residue of a substrate. This space is occupied by the rhamnose ring in the complex of phosphoramidon with thermolysin (Weaver et al., 1977) and by various aromatic groups in dipeptide–inhibitor complexes (Kester & Matthews, 1977). However, if bidentate ligation was involved, more atoms of the inhibitor would be brought closer to the zinc atom and it is possible that a methyl group would then begin to make steric contact with some portion of thermolysin's surface, interfering with the binding. An X-ray crystallographic study of thermolysin complexed with HONH-Bzm-L-Ala-Gly-NH_2 is currently underway (B. Matthews and M. Holmes, private communication). The hydroxamic acid functional group is interacting with the zinc atom in the complex, but at present the exact nature of the zinc ligation is not certain.

The *N*-hydroxy peptide structure has one unique property: unlike the hydroxamic acids, replacement of the formyl or acetyl groups by an amino acid residue or a peptide residue would result in a peptide structure containing the potential zinc ligand in the interior rather than at one end or the other. Such an inhibitor structure might be essential for other metalloproteases such as collagenase which probably need to recognize amino acid residues on either side of the catalytic site. In order to test the possible usefulness of such a structure, we synthesized Boc-Gly-HOLeu-NH_2, which is a relative of good thermolysin substrates such as furylacrylyl-Gly-L-Leu-NH_2. Unfortunately, the *N*-hydroxy peptide did not bind to thermolysin (K_I > 5 mM), again probably due to steric problems.

Bestatin and Amastatin. Umezawa and co-workers have isolated two aminopeptidase inhibitors from culture filtrates of actinomycetes. Bestatin [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-Leu-OH] inhibits both leucine aminopeptidase (K_I = 0.02 μM) and aminopeptidase B (K_I = 0.06 μM) (Suda et al., 1976; Nishizawa et al., 1977), while amastatin [(2*S*,2*R*)-3-amino-2-hydroxy-5-methylhexanoyl-L-Val-L-Val-L-Asp-OH] inhibits leucine aminopeptidase (IC_{50} = 0.5 μg/mL) and aminopeptidase A (IC_{50} = 0.54 μg/mL) (Aoyagi et al., 1978). Both bestatin and amastatin contain an α-hydroxycarbonyl functional group attached to the carbon bearing a side chain most likely to be recognized by the enzyme's primary substrate-binding site, which is S_1 in the case of aminopeptidase.

Two compounds containing the α-hydroxycarbonyl group were synthesized and examined as thermolysin inhibitors. The first, glycolyl-L-Phe-L-Ala-Gly-NH_2, was a poor inhibitor (K_I = 2.9 mM). The second, Z-$NHCH_2CHOHCO$-L-Leu-NH_2, resembles both bestatin and the good thermolysin substrate Z-Gly-Leu-NH_2. This is also a poor inhibitor (K_I > 5 mM). We then perceived a distinct difference between bestatin and our inhibitors. In the case of the aminopeptidases, the positioning side chain (a benzyl group in bestatin) probably interacts with the S_1 subsite of the enzyme. This places the α-hydroxycarbonyl group in the same location as the peptide bond of a substrate and in position to coordinate with the zinc atom (Figure 4a) in much the same manner as that proposed for hydroxamic acids (Figure 4b) and *N*-hydroxy peptides (Figure 4c). However, in the case of thermolysin, the positioning side chain interacts with the S_1' subsite of the enzyme and the α-hydroxycarbonyl group of our two inhibitors would be one atom removed from the proper placement for coordination (Figure 4d). This also explains why acetoacetyl-L-Phe-L-Ala-Gly-NH_2 is a poor inhibitor (K_I = 3.1 mM) in spite of having an excellent coordinating functional group. Investigation of compounds containing one less atom between the P_1' side chain and the α-hydroxycarbonyl group as potential thermolysin inhibitors would be well worth the effort, but their synthesis would involve more difficulty.

Affinity Chromatography. The affinity resin NONH-Bzm-L-Ala-Gly–aminopropylagarose proved to be quite effective and useful for purification of thermolysin and the *B.*

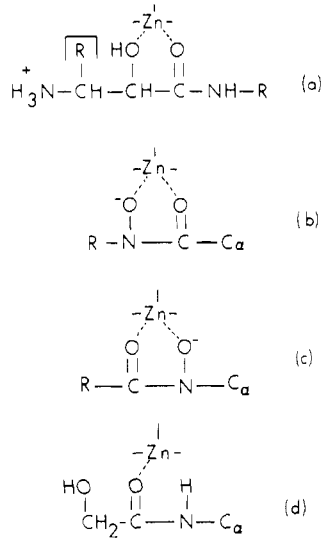

FIGURE 4: Schematic diagram showing the proposed interaction between the active site of (a) an aminopeptidase and bestatin or amastatin or between thermolysin and (b) a hydroxamic acid, (c) an *N*-hydroxy peptide, and (d) the glycolyl dipeptide. In the case of the aminopeptidase, the positioning is determined by the S_1–P_1 interaction (left side of the zinc atom). With thermolysin the positioning is determined by the S_1'–P_1' interaction (right side of the zinc atom).

subtilis neutral proteases. Several other adsorbents have been used with a variety of metalloproteases. A protease from *Clostridium histolyticum* was purified by using agarose–caproyl-Gly-Leu-OH (Sparrow & McQuade, 1973). The adsorbent could only be used 3 or 4 times due to the cleavage of the Gly–Leu bond by the enzyme. Our hydroxamic acid adsorbent on the other hand has been used over 50 times with a variety of enzymes with no noticeable loss of potency. D-Phenylalanine has been used as a ligand for the affinity chromatography of thermolysin and the separation of the *B. subtilis* neutral proteases A and B (Pangburn et al., 1973). This adsorbent, however, also binds serine proteases such as chymotrypsin and subtilisin, in contrast to the results which we observed. In addition, D-Phe is not a strong inhibitor of the *B. subtilis* neutral proteases and the conditions utilized for elution of these metalloproteases indicate that they are much more tightly bound to the hydroxamic acid adsorbent. Recently, an adsorbent has been reported which contains phosphoramidon as the ligand (Komiyama et al., 1975a). Thermolysin binds to this column at pH 7.0 and is eluted at pH 9.0. A neutral protease from *B. subtilis* var. *amylosaccariticus* was also eluted as a single peak. Phosphoramidates offer most of the advantages of the hydroxamic acids as ligands for affinity chromatography. In fact, an analogue of phosphoramidon has been attached to agarose and used in the purification of the neutral endoprotease from *B. cereus* (Holmquist, 1977). The choice between a phosphoramidate or a hydroxamic acid ligand for use with a new metalloprotease would probably be determined by the respective ease of synthesis of the two ligands.

Conclusion

In this paper we have synthesized a number of thermolysin inhibitors which carry different functional groups and have shown that the appropriate hydroxamic acids, *N*-hydroxy peptides, thiols, and phosphoramides are quite useful as inhibitors. The relative binding efficiency of the various functional groups is determined by a combination of their ligating ability and their resemblance to the transition state for peptide bond hydrolysis. Also, we have reported the synthesis of an affinity adsorbent which contains the $HONHCOCH(CH_2C_6H_5)CO$-L-Ala-Gly moiety. The adsorbent was shown to be specific for several zinc metalloendoproteases, and a variety of conditions were developed for elution of the enzymes from the column. The sequence of the inhibitors corresponds to the P_1'–P_3' residues of a good thermolysin substrate and could easily be altered to match the preferred sequences in substrates of other metalloproteases. We are currently preparing inhibitors for other metalloproteases to utilize in the study of their physiological function and in their purification by affinity chromatography.

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Supplementary Material Available

Experimental details for the synthesis of the new compounds reported (8 pages). Ordering information is given on any current masthead page.

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Stereospecificity of the Metal–Adenosine 5′-Triphosphate Complex in Reactions of Muscle Pyruvate Kinase†

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ABSTRACT: Rabbit muscle pyruvate kinase in the presence of mono- and divalent cations catalyzes the phosphorylation of glycolate by $Cr^{III}ATP$. The product complex CrADP–glycolate-P is released slowly from the enzyme, and generally only a single turnover is observed for these reactions. The Λ β,γ-bidentate isomer and the four α,β,γ-tridentate isomers are all inactive as substrates in this reaction. Only the Δ isomer of β,γ-bidentate CrATP is active in the phosphoryl transfer reaction and is the most active isomer in the enolization of pyruvate catalyzed by pyruvate kinase. In contrast to the phosphoryl transfer reaction, all of the bidentate and tridentate CrATP isomers are effective in promoting the pyruvate kinase catalyzed enolization of pyruvate. The V_{max} of the most active isomer Δ β,γ-bidentate CrATP (Mn^{2+} and K^+; pH 5.7; 21 °C) is 6.0 μmol of pyruvate enolized per min mg of protein, and the K_m is 100 μM. In comparison the V_{max} of the Λ bidentate isomer under the same conditions is 3-fold lower and the K_m is 1.7-fold greater. The four tridentate isomers of CrATP do not differ greatly in their activity but are all less active than the bidentate isomers by more than 1 order of magnitude. The most active tridentate isomer at pH 5.7 has a V_{max} of 0.26 μmol of pyruvate enolized per min mg of protein and a K_m of 600 μM. Pyruvate kinase also requires an enzyme-bound divalent cation for activity. The order of activation by various enzyme-bound divalent cations in the phosphorylation of glycolate by CrATP is the same as it is in the CrATP-stimulated enolization of pyruvate: Mn(II) > Co(II), Zn(II) > Mg(II) ≫ Ca(II) ~ 0. The paramagnetic effects of the bidentate CrATP isomers on the relaxation rate of water protons are significantly lower than those of the tridentate isomers. Analysis of the temperature dependence of these effects indicates 1 order of magnitude slower escape of protons from the coordination sphere of bidentate CrATP (1.1×10^6 s^{-1}) than that from tridentate CrATP, suggesting an intramolecular hydrogen-bond interaction in the bidentate isomers but not in the tridentate isomers.

Rabbit muscle pyruvate kinase catalyzes the reversible phosphorylation of ADP by 2-phosphoenol pyruvate and, in a second step, the reversible protonation of the pyruvate enolate (Rose, 1960; Robinson & Rose, 1972). Previous studies of the role of metal ions in the pyruvate kinase reaction, by a variety of kinetic and magnetic resonance techniques, have revealed the requirement for two divalent cations per active site (Gupta et al., 1976a,b; Gupta & Mildvan, 1977). One of these divalent cations binds to the enzyme and forms second-sphere complexes with the bound substrates while the second binds directly to the triphosphate chain of the ATP (Gupta et al., 1976a,b; Gupta & Mildvan, 1977). The kinetic and magnetic resonance studies established coordination of the β- and γ-phosphoryl groups of enzyme-bound ATP by the nucleotide-bound metal but were inconclusive as to α-coordination (Gupta & Mildvan, 1977). The present studies were undertaken to resolve this ambiguity and to study, by an entirely independent method, the chelate structure and stereochemistry of the active metal–ATP complex on pyruvate kinase.

A direct and independent approach to this problem is that used previously by Cornelius & Cleland (1978) to define the active metal–ATP isomer in the yeast hexokinase reaction

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