Inhibition of Leucine Aminopeptidase by Amino Acid Hydroxamates*

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Amino acid hydroxamates are strong competitive inhibitors of leucine aminopeptidase from porcine kidney. The side chain specificity for inhibition correlates well with substrate specificity. L-Leucine hydroxamate $(K_i = 14 \mu M)$ protects the enzyme from inactivation by EDTA and is presumed to be a bidentate ligand of the zinc at the active site. A substituted $\beta$-mercaptoketone which may bind in a similar way is also a potent inhibitor $(K_i = \sim 1 \mu M)$. The binding of these inhibitors suggests a mechanism for this enzyme in which a zinc-bound hydroxide ion participates in concerted proton-transfer processes, while the coordination and charge field at the zinc atom remain unchanged.

In recent years, considerable attention has been given to the structure and mechanism of zinc proteases. The three-dimensional structures of some of these enzymes (e.g. carboxypeptidase and thermolysin) have been determined (1, 2) and extensive kinetic studies have been performed (3). Other zinc proteases have not been characterized to the same degree but are of great physiological interest (e.g. enkephalinase and the angiotensin-converting enzyme). The work of Byers and Woffenden (4) has stimulated the design of a variety of tight-binding inhibitors for these enzymes (5–7). Such inhibitors can be useful not only in mechanistic studies but also in the characterization of enzymes, in affinity chromatography, in determining the role of enzymes in vivo, and, perhaps most significantly, in clinical treatment.

Leucine aminopeptidase from porcine kidney has been characterized as a zinc exopeptidase of comparatively large molecular weight (8). Its substrate specificity has been clearly established (9), but its mechanism has not been investigated in great detail (10). We report here the inhibition of this enzyme by amino acid hydroxamates and related compounds. From a consideration of the binding of these inhibitors, we propose a reaction mechanism in which a zinc-bound water molecule alternates as proton donor and acceptor.

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MATERIALS AND METHODS

Microsomal leucine aminopeptidase (specific activity, 13.3 units/mg) was obtained from Sigma as a suspension in ammonium sulfate. L-Leucine- $p$-nitroanilide, N-CBZ-$\beta$-phenylalanine chloromethyl ketone, and various amino acid derivatives (including hydroxamates) were also purchased from Sigma.

Aminopeptidase activity was measured by monitoring the increase in absorbance at 400 nm using L-leucine-$p$-nitroanilide as substrate (11). The commercially supplied enzyme (5 mg/ml) was diluted 50-fold into 0.1 M Tris-HCl, pH 7.5, containing 0.01 mM ZnCl$_2$ and 0.2 mg/ml of bovine serum albumin. Aliquots (20 ml) were assayed in 1.0 ml of Tris-HCl buffer (0.1 M, pH 7.5) at 25°C. For the determination of dissociation constants of the inhibitors, several substrate concentrations ranging from 0.2 to 1 mM $(K_i = 0.5 \mu M)$ and several inhibitor concentrations in the neighborhood of the $K_i$ value were employed. The inhibitors were dissolved in a small amount of 10% acetic acid and then diluted to a 2 mM stock solution in the assay buffer. The data were evaluated by Dixon plots.

In the inactivation experiments, the enzyme (40 mg/ml) was incubated at 37°C in 0.1 M Tris-HCl buffer, pH 7.5, containing 10 mM EDTA and L-leucine hydroxamate ranging in concentration from 0 to 0.08 mM. At various time intervals, aliquots (50 ml) were withdrawn and added to 1.0-ml assay mixtures at 25°C. The extent of inactivation during the assay was insignificant, presumably due to a reduction in the EDTA concentration, a decrease in temperature, and the presence of substrate.

For the synthesis of the mercaptoketone inhibitor (1) (see Fig. 2), N-CBZ-phenylalanine chloromethyl ketone (0.61 mmol) was refluxed for 4 h in 5.7 ml of absolute ethanol with potassium hydroxide (0.67 mmol) and thiouctic acid (0.77 mmol). Methylene chloride (5 ml) was added, the mixture was filtered to remove KCI, and the filtrate was evaporated. The residue was recrystallized from absolute ethanol (white needles, m.p. 100°C). NMR showed all the expected proton resonances for S-acetyl-2-keto-3-carbobenzyloxyamido-4-phenylbutanethiol ester.

The S-acetyl and N-CBZ groups were hydrolyzed off in 30% HBr in acetic acid at room temperature for 20 h. After dilution with water, the mixture was extracted with methylene chloride to remove benzyl alcohol and acetic acid and was evaporated to dryness. The residual oil could not be crystallized, but its infrared spectrum was consistent with the structure (I) and showed characteristic frequency of amino (3400 cm$^{-1}$) and carbonyl (1725 cm$^{-1}$) groups. The presence of the expected amounts of free thiol and free amino groups was determined by reaction with 5,5'-dithiobis-2-nitrobenzoic acid and trinitrobenzene sulfonic acid, respectively. The color yields were measured at 412 and 335 nm, respectively, and were compared to mercaptoethanol and ethanolamine standards.

RESULTS AND DISCUSSION

Structural Requirements for Inhibition—A number of amino acid hydroxamates are found to be strong competitive inhibitors of leucine aminopeptidase (Table I). Inhibition of the enzyme occurs with no detectable time-lag and is fully reversible upon dilution. The effectiveness of inhibition appears to depend on the presence of an extended side chain. Thus, L-Leu-NHOH is more than 100 times as potent as Gly-NHOH. The corresponding amide (L-Leu-NH$_2$), alcohol (L-leucinol), and hydrazide (L-Tyr-NHNH$_2$) as well as the free amino acid are bound much less tightly.

The extensive work of Nishino and Powers (5, 12) has shown that peptides containing hydroxamate groups at either

The abbreviation used is: CBZ, carbobenzyloxy.
the COOH or NH₂ terminus are potent inhibitors of thermolysin and the zinc-containing elastase from *Pseudomonas aeruginosa* but are ineffective towards leucine aminopeptidase. Conversely, L-Leu-NHOH, which is shown here to be a good inhibitor of aminopeptidase, was reported to be much less effective (Kᵢ = 190 μM) towards thermolysin (5). Since thermolysin and *P. aeruginosa* elastase are endopeptidases whereas the present enzyme is an NH₂-terminal α-xopeptidase, the above differences are readily attributable to the specificity in substrate recognition. A proline derivative containing a hydroxamate moiety at the NH₂-terminus is also a powerful inhibitor of the zinc-containing angiotensin-converting enzyme (13) although not as potent as captopril (d-3-mercaptopropanoyl-l-proline).

**Probable Mode of Binding**—The general effectiveness of hydroxamates as inhibitors of zinc proteases and our finding that the corresponding amides, hydrazides, and alcohols are bound much more weakly suggest that the hydroxamate anion binds to the metal in a bidentate manner. Recently, Holmes and Matthews (14) have established that hydroxamate inhibitors provide two of the ligands in a pentacoordinated zinc complex in thermolysin. Since our enzyme is not available in a crystalline form for x-ray diffraction studies, we have attempted to probe indirectly its interaction with hydroxamates. We have found that L-leucine hydroxamate effectively protects the enzyme from inactivation by EDTA (Fig. 1). The kinetics of inactivation is strictly first order, and the concentration of inhibitor for half-maximal protection (12 μM) is in good agreement with the Kᵢ determined from inhibition of activity. Extrapolation to infinite inhibitor concentration indicates complete protection of the enzyme. Although the effect of any ligand on the reactivity of any part of the protein may be indirect (i.e. mediated by the polypeptide backbone), the complete stabilization of zinc is highly suggestive of a direct interaction.

In attempting to understand why hydroxamates are particularly good inhibitors, we rely on the accumulated evidence in favor of pentacoordinated zinc as a reaction intermediate in zinc enzymes ranging from carboxic anhydrase (15) and alcohol dehydrogenase (16) to proteases (14, 17). In these intermediates, the protein provides three of the ligands with substrate contributing a carbonyl moiety. The fifth ligand is invariably a water molecule or hydroxide ion (18). The hydroxamate inhibitors, therefore, combine in one molecule the binding capabilities of both the substrate and the hydroxide ion (Fig. 2a). We have attempted to test the postulated mode of interaction by synthesizing the β-mercaptoketone derivative shown in Fig. 2b. The commercially available chymotrypsin-inactivator N-CBZ-l-phenylalanine chloromethyl ketone was allowed to react with potassium thioacetate to yield the acetylthiol ester derivative (CBZ-NH-CH(CH₃CH₂)-CO-CH₂-S-COCH₃). Hydrolysis in 30% HBr in acetic acid gave the expected product (f) which was at least 10 times as potent an inhibitor (Kᵢ ≈ 1 μM) as the corresponding hydroxamate. Substrate analogs containing thiol groups have provided some of the most effective inhibitors of zinc proteases (6, 12, 19). Further work will be required to determine whether β-mercaptoketones offer any advantages as an alternative class of inhibitors.

**Proposed Mechanism for Leucine Aminopeptidase**—Consideration of the mode of binding of hydroxamates to leucine aminopeptidase has focussed attention on the role of the postulated zinc-bound hydroxide ion. In the proposed mechanisms for carboxypeptidase A, thermolysin, and carboxic anhydrase, the hydroxide ion in the pentacoordinate zinc complex has been assigned the role of a nucleophile. However, we favor a mechanism in which this fifth ligand operates alternately as a general base and a general acid. A similar role for zinc-bound water has also been proposed for alcohol dehydrogenase (16).
Hydroxamates as Inhibitors of Leucine Aminopeptidase

By analogy with other zinc proteases, we assume that the free enzyme contains a hydroxide ion in the tetracoordinated zinc complex. The binding of the substrate generates the pentacoordinated structure shown in Fig. 3a. We postulate a concerted proton-transfer process in which the hydroxide ion acts as a general base leading to the attack (by another molecule of water) on the carbonyl of the scissile peptide bond and formation of the tetrahedral intermediate. Subsequent breakdown of this intermediate is facilitated by the zinc-bound water molecule which now acts as a proton donor (Fig. 3b). Diffusion of the two products (Fig. 3c) from the active site regenerates the original form of the enzyme without requiring further transformations. The central feature of this mechanism is that, in the most critical steps (the formation and breakdown of the tetrahedral intermediate), the coordination of zinc and the net charge field around it remain essentially unchanged. There are also no major movements of atoms. The associated energy barriers are, therefore, expected to be relatively small. Our proposal is distinct from the mechanism of Bryce and Rabin (10), in which the role of zinc is simply to polarize the carbonyl of the scissile bond.

It should be emphasized that the above scheme does not imply that amino acid residues do not participate in the mechanism. Previous work on the bovine lens enzyme has demonstrated that histidine is essential for activity (20). It is extremely likely that the two water molecules are hydrogen bonded to other groups in the active site such as carboxylates and imidazoles. The resulting immobilization of these water (or hydroxide) molecules and the fine tuning of their proton affinity may facilitate the catalytic process. However, if one or more of these amino acid side chains would directly donate or accept a proton, the modified mechanism would require a further isomerization step at the end to regenerate the active form of the enzyme. It is also possible that, for stereochemical reasons, an extra water molecule may be interposed between the amide nitrogen and the attacking water molecule shown here. While many details remain unclear, the idea of two ligands in a pentacoordinated zinc complex alternating between charged and uncharged forms is appealing in our opinion and may find application in the hydrolysis of peptide bonds by other zinc enzymes. In particular, the proposed mechanism is consistent with the pairing principle of bond rupture and formation recently enunciated by Green and Vande Zande (21).

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(a) 
(b) 
(c) 

Fig. 3. Proposed mechanism of leucine aminopeptidase-catalyzed peptide hydrolysis.