Hydroxamates and Aliphatic Boronic Acids: Marker Inhibitors for Aminopeptidase†

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ABSTRACT: Metal-coordinating inhibitors of *Aeromonas* aminopeptidase (EC 3.4.11.10) were paired with either a transition state analogue inhibitor or a hydrophobic-site inhibitor in multiple-inhibition studies designed to determine the proximity of the respective binding loci. The specificity of the aminopeptidase toward the N-terminal side chain was established by determining K_i values for amino acid amides, used as alternate-substrate inhibitors of the hydrolysis of L-leucine *p*-nitroanilide; the enzyme showed a strong preference for binding of the amides of leucine, isoleucine, and phenylalanine. L-Amino acid hydroxamates, known to form bidentate ligands with the enzyme-bound zinc in other enzymes, proved to be good inhibitors of *Aeromonas* aminopeptidase, a metalloenzyme that contains 2 mol of zinc/mol. Smaller hydroxamate compounds were bound approximately 4000 times more tightly than analogous amides, and the hydroxamates of leucine, isoleucine, and phenylalanine had much lower (50–100 times) binding constants than their analogous amides. The effectiveness of the hydroxamido group was evident from the observation that formyl hydroxamate—a compound without a hydrophobic moiety—exhibited a K_i equal to that of isoleucine amide. 1-Butaneboronic acid, a competitive inhibitor of *Aeromonas* aminopeptidase that functions by forming an analogue of the transition state, was used as a marker for the catalytic subsite in multiple-inhibition studies, and hydroxamate inhibitors of varying sizes were used as markers for the enzyme-bound zinc. Isoamyl alcohol, structurally similar to the side chain of preferred substrates, was chosen as a hydrophobic binding site marker. Mutual exclusivity of binding was observed between the following pairs of inhibitors: 1-butaneboronic acid and L-leucine hydroxamate; L-leucine hydroxamate and formyl hydroxamate; formyl hydroxamate and 1-butaneboronic acid; 1-butaneboronic acid and boric acid. The fact that binding of formyl hydroxamate completely blocked binding of both L-leucine hydroxamate and 1-butaneboronic acid demonstrated that the mutual exclusivity between the latter two inhibitors was not due solely to competition between the hydrophobic side chains and that the dihydroxyboron group of 1-butaneboronic acid and the hydroxamido group of the hydroxamates cannot bind simultaneously. Thus, the binding loci for these groups on the enzyme are in close proximity. Simultaneous binding was observed with the pairs isoamyl alcohol and formyl hydroxamate, isoamyl alcohol and hydroxylamine, and isoamyl alcohol and boric acid, but complete interference occurred between isoamyl alcohol and acetohydroxamic acid. The ability of formyl hydroxamate—but not acetohydroxamate—to bind simultaneously with the hydrophobic inhibitor implies that the hydrophobic pocket of the enzyme is separated from the binding sites for polar groups by no more than the dimensions of a methylene group. The interactions observed between the various inhibitors demonstrate that the essential zinc ion of *Aeromonas* aminopeptidase is located within, or very near, the catalytic subsite that binds the substrate carboxamido group. This strongly suggests that the metal ion (or a water molecule/hydroxyl ion coordinated to it) is one of the enzyme functional groups that interact directly with the substrate atoms surrounding the scissile bond.

Nishino & Powers (1978) designed a number of metalloprotease inhibitors on the basis of both the specificity requirements of thermolysin, the model enzyme they used, and the functional groups needed to bind the inhibitors to the zinc in the active site. Peptide thiosemicarbazides proved to be ineffective, and peptide hydrazides produced only weak inhiibition of thermolysin; however, peptide hydroxamic acids and *N*-acyl-*N*-hydroxy peptide compounds proved to be potent inhibitors with K_i values in the micromolar range. In an extension of this work, Rasnick & Powers (1978) chemically modified Glu_{143} of thermolysin with the active site directed *N*-hydroxy peptide *N*-(chloroacetyl)-DL-*N*-hydroxyleucine methyl ester. Subsequently, hydroxamic acid, thiol, phosphoramidate, and *N*-hydroxy peptide derivatives with the same peptide backbone were prepared by Nishino & Powers (1979), and a comparison was made of their ability to inhibit thermolysin. Although phosphoramidon, a transition state analogue inhibitor of thermolysin (Weaver et al., 1977), might be expected to be the most potent of these inhibitors, thiol and hydroxamido derivatives provided slightly more effective inhibition. The stability and usefulness of the hydroxamido group was illustrated by a successful affinity chromatography system utilizing a hydroxamate ligand, devised for purifying thermolysin and *Bacillus subtilis* neutral proteases (Nishino & Powers, 1979). *Pseudomonas aeruginosa* elastase, another metalloendopeptidase, was strongly inhibited by a hydroxamido group attached to peptides that fulfilled the requirements of the extended substrate binding site of this enzyme (Nishino & Powers, 1980). These results supported the original suggestion by Nishino & Powers (1978) that hydroxamates should have a general applicability as inhibitors of metalloproteases. They later pointed out that, whereas the thiol and phosphoro groups form monodentate ligands to the catalytic zinc, hydroxamates could form bidentate ligands with both oxygens participating (Nishino & Powers, 1979). X-ray crystallographic studies by Holmes & Matthews (1981) showed that both an amino acid hydroxamate and an *N*-hydroxy peptide formed bidentate ligands in the pentacoordinate zinc complex with thermolysin.

Metalloenzymes are of widespread occurrence among the aminopeptidases, as well as neutral proteases, and we have shown that the activity of *Aeromonas* aminopeptidase (EC 3.4.11.10) is dependent upon the presence of the zinc ions

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found in this enzyme (Prescott & Wilkes, 1976). We have been investigating, by kinetics (Baker & Prescott, 1980) and chemical modifications (Mäkinen et al., 1982a,b), the active site and mechanism of action of this enzyme, selected as a model for aminopeptidases because of its small size, single-peptide structure, and high degree of stability (Prescott & Wilkes, 1976). The fact that hydroxamates bind to the active site zinc in metalloendopeptidases suggested that it would be possible to probe the active site of *Aeromonas* aminopeptidase by means of these inhibitors. Studies of the binding of inhibitors to enzyme active sites are particularly informative if the inhibitors resemble substrates (or parts of substrates) and if it can be shown that the inhibitors bind in a manner analogous to the binding of substrates. The hydroxamate compounds used in this study as inhibitors of *Aeromonas* aminopeptidase include a series of amino acid hydroxamates ($H_2NCHRCONHOH$); it was therefore of considerable interest to us to obtain accurate binding data for the corresponding series of amino acid amide substrates. Wagner et al. (1972) screened a number of amino acid amides as substrates of *Aeromonas* aminopeptidase. In that study, however, the enzyme reaction was measured by monitoring the decrease in the peptide-bond absorbance at 230 nm; this relatively insensitive assay method necessarily limited the determination of K_m and k_{cat} to the more rapidly hydrolyzed, "preferred" substrates of the aminopeptidase, which were found to be those with bulky, hydrophobic side chains. In the present study, we wished to compare the binding of a wider variety of amino acid hydroxamates with binding of the analogous amides. Therefore, we devised experiments in which amino acid amides were used as alternate-substrate inhibitors (Dixon & Webb, 1958; Wharton & Eisenthal, 1981; Rohrbach et al., 1981) of the hydrolysis of L-leucine *p*-nitroanilide by the aminopeptidase, thereby making it possible to obtain binding data for amino acid amides with a wide range of side-chain sizes, including some that are hydrolyzed too slowly to permit direct determination of K_m. We then determined inhibition constants for a variety of L-amino acid hydroxamates, as well as several small aliphatic hydroxamates, in order to evaluate the relative contributions of the zinc-marker functional group and the hydrophobic side chain of these compounds to binding.

Among other inhibitors that we have characterized for *Aeromonas* aminopeptidase, two types—boronic acids and aliphatic alcohols—offered advantages for this study. 1-Butaneboronic acid (hereafter BuBA)[1] is an excellent inhibitor for this enzyme (Baker & Prescott, 1980) because it reacts with the enzyme to form a transition-state analogue and is thus a marker for the catalytic subsite. Several aliphatic alcohols have been found to inhibit this aminopeptidase (DeShazo, 1968), and we used one of these, isoamyl alcohol, as a hydrophobic binding site marker. By using various combinations of these inhibitors, we evaluated the degree of overlap between the binding sites in the aminopeptidase for those compounds that inhibit by binding zinc, those that inhibit by forming transition-state analogues, and those that bind predominantly to the hydrophobic site.

Materials and Methods

Materials. L-Valine amide, L-tyrosine amide, L-phenylalanine amide, acetohydroxamic acid, glycine hydroxamate, L-leucine hydroxamate, L-tyrosine hydroxamate, L-lysine hydroxamate, and L-tryptophan hydroxamate were purchased from Sigma Chemical Co. L-Alanine amide, L-norleucine amide, L-isoleucine amide, and L-tryptophan amide were obtained from Vega Chemical Co. L-Methionine amide, L-leucine amide, Boc-L-phenylalanine *N*-hydroxysuccinimide ester, and Boc-L-valine *N*-hydroxysuccinimide ester were Bachem products; BuBA, phenylboronic acid, and triethylamine were purchased from Aldrich Chemical Co. Tricine buffer and glycine amide were from Calbiochem, and hydroxylamine hydrochloride was from Fisher. Mallinckrodt was the source of isoamyl alcohol, and Specpure $ZnSO_4 \cdot 7H_2O$ was from Johnson-Matthey Co. Amino acid hydroxamates prepared by Enzyme Systems Products, Livermore, CA, were L-valine hydroxamate, L-threonine hydroxamate, L-isoleucine hydroxamate, and L-phenylalanine hydroxamate.

Enzyme Assays. Aeromonas aminopeptidase was isolated in this laboratory from culture filtrates of *Aeromonas proteolytica* by procedures described by Prescott & Wilkes (1976). The purified aminopeptidase had a specific activity of 130 μmol min^{-1} (mg of protein)$^{-1}$ for 0.5 mM L-leucine *p*-nitroanilide as substrate at 25 °C in 20 mM Tricine buffer, pH 8.0, containing 0.2 M KCl and 0.1 mM $ZnSO_4$. Enzyme dilutions of 10^{-7} M were stored frozen in 1 mM Tricine that was 0.2 M in KCl and 0.1 mM in $ZnSO_4$. L-Leucine *p*-nitroanilide, at concentrations between 0.1 and 0.02 mM, was used for all activity measurements in the buffer described above. The increase in absorbance at 405 nm was monitored on a Zeiss PMQII or a Varian Model 219 spectrophotometer and with the value of $\Delta\epsilon_{405}$ = 10 800 (Tuppy et al., 1962). Under typical assay conditions in which the enzyme concentration was 9×10^{-10} M, no more than 10% of the substrate was hydrolyzed. Hydroxamate compounds were dissolved and stored in 0.1 mM acetic acid, pH 4.5, and activity determinations at four concentrations of hydroxamate were made for each of five substrate concentrations. Double-reciprocal Lineweaver–Burk plots were constructed, and the slope of each line was plotted against the concentration of inhibitor; a linear-regression program furnished with a Hewlett-Packard 65 calculator was used to determine the intercept (=$-K_i$). Multiple-inhibition experiments involving simultaneous inhibition by two different inhibitors were conducted at a fixed substrate concentration of 0.0375 mM L-leucine *p*-nitroanilide. Inhibitor I was added to the reaction mixture at levels of 0, 2.5 and 5 times K_i and inhibitor II was added at four concentrations between 0 and 10 times K_i for each of the three levels of inhibitor I.

Synthesis of Formyl Hydroxamate. N-Hydroxyformamide or formylhydroxamic acid was prepared by the reaction of ethyl chloroformate (0.84 mL or 10 mmol) with hydroxylamine hydrochloride (0.695 g or 10 mmol) in 2.5 mL of absolute ethanol by the method of Fishbein et al. (1969). Two milliliters of 10 N alcoholic NaOH was added to the above mixture with stirring while the temperature was maintained at 5 °C. After 30 min, the mixture was adjusted to pH 6.0 by the addition of approximately 0.70 mL of concentrated HCl. The solvent was evaporated under vacuum, two 10-mL portions of absolute ethanol were added and evaporated under vacuum to remove traces of water, and four 10-mL portions of warm ethyl acetate were used to solubilize the formyl hydroxamate that crystallized upon cooling. The product exhibited a melting point of 80 °C compared with the published value of 79 °C (Fishbein et al., 1969), and the hydroxamate content was found by reaction with ferric chloride (Lippman & Tuttle, 1945) to contain the expected amount. The same product was synthesized by performing the reaction in aqueous solution and in dimethylformamide.

[1] Abbreviations: Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Boc, *tert*-butyloxycarbonyl; BuBA, 1-butaneboronic acid.

Table I: Inhibition of *Aeromonas* Aminopeptidase by Amides, Alcohols, and Boronic Acids

inhibitor	K_i (M)	inhibitor	K_i (M)
formamide	1.5	L-tyrosine amide	7.2×10^{-3}
glycine amide	2.0×10^{-1}	L-tryptophan amide	2.0×10^{-2}
L-alanine amide	1.3×10^{-1}	L-valine	1.9×10^{-3}
L-valine amide	7.5×10^{-4}	L-leucine	8.0×10^{-4}
L-threonine amide	7.7×10^{-2}	L-isoleucine	7.2×10^{-4}
L-methionine amide	3.1×10^{-3}	boric acid	1.3×10^{-1}
L-leucine amide	7.0×10^{-4}	methylboronic acid	4.1×10^{-2}
L-isoleucine amide	1.3×10^{-4}	1-butaneboronic acid	9.6×10^{-6}
L-norleucine amide	6.7×10^{-4}	phenylboronic acid	4.0×10^{-4}
L-phenylalanine amide	4.7×10^{-4}	isoamyl alcohol	1.35×10^{-3}

Synthesis of Amino Acid Hydroxamates. The hydroxamic acids of L-alanine, L-isoleucine, and L-phenylalanine were prepared from their respective Boc *N*-hydroxysuccinimide esters by a procedure described by Nishino & Powers (1978) for the preparation of *N*-carbobenzoxy-blocked peptide hydroxamates. In the preparation of L-phenylalanine hydroxamate, 5 mL of dioxane was used to dissolve 726 mg (2 mmol) of Boc-L-phenylalanine *N*-hydroxysuccinimide ester, and to this was added 0.138 g (2 mmol) of hydroxylamine hydrochloride in 14 mL of dimethylformamide to which had been added 0.28 mL (2 mmol) of triethylamine. The mixture was stirred overnight, filtered, and evaporated to dryness under reduced pressure. The Boc-L-phenylalanine hydroxamate was extracted in warm ethyl acetate; the salts were removed by filtration; the ethyl acetate layer was washed first with 4% HCl and then with 4% sodium bicarbonate before being dried with $MgSO_4 \cdot 7H_2O$. Crystals of Boc-amino acid hydroxamate were dried under vacuum. Deblocking was accomplished by treatment with 4 N HCl in dioxane for 30 min after which the crystalline amino acid hydroxamate hydrochloride was washed with cold dioxane and dried under vacuum over NaOH. The hydroxamates of L-alanine and L-isoleucine were prepared similarly, but because of their solubility, the blocked amino acid hydroxamates were separated by several extractions with warm ethyl acetate and were not subjected to the washing procedure. The extracts were evaporated, cooled, and filtered; crystallization from this filtrate yielded the pure blocked hydroxamate. Preparations of L-isoleucine hydroxamate and L-phenylalanine hydroxamate from this laboratory and those purchased from Enzyme Systems Products exhibited identical inhibition of activity.

Results

Earlier work (Wagner et al., 1972) showed that several amino acid amides are susceptible to hydrolysis by *Aeromonas* aminopeptidase. In the present experiments, however, we used L-amino acid amides as alternate-substrate inhibitors of the hydrolysis of L-leucine *p*-nitroanilide by the aminopeptidase. The high sensitivity of the L-leucine *p*-nitroanilide assay permitted the use of enzyme concentrations that resulted in the hydrolysis of less than 1% of the amino acid amide while less than 10% of the L-leucine *p*-nitroanilide was being cleaved. The alternate-substrate inhibitor approach also allowed us to measure the binding of some L-amino acid amides that are hydrolyzed too slowly to permit direct K_m determinations when these amides are used as the monitored substrate. From the data in Table I, it is apparent that the larger and more hydrophobic residues are the most tightly bound to the active site. That the strength of binding is primarily controlled by the hydrophobicity of the side chain, rather than by the nature of the polar functions attached to the carbonyl moiety, is confirmed by the fact that the corresponding amino acids were found to have K_i values in the millimolar range similar to those of the amides. The same conclusion is also indicated by the K_i value of 1.35 mM for isoamyl alcohol, which has a side chain structurally similar to that of leucine. Additional insight regarding the effects of side-chain polarity can be gained by comparing the K_i values for the amides of threonine and valine and for the amides of phenylalanine and tyrosine. The relatively poor efficiency of tryptophan amide and methionine amide as inhibitors is probably ascribable to their being too large to fit into the hydrophobic pocket. Weak inhibition was observed with boric acid and methylboronic acid, and stronger binding resulted with phenylboronic acid. BuBA, as observed by Baker & Prescott (1980), was a very good inhibitor of this aminopeptidase. The values in Table II show that L-amino acid hydroxamates are much more tightly bound by *Aeromonas* aminopeptidase than the analogous amino acid amides. In contrast to the amides, even the small hydroxamic acids are good inhibitors; the enhancement of inhibition by the hydroxamido group is evident from a comparison of formamide, glycine amide, and alanine amide with the corresponding hydroxamic acids, which reveals differences of 3000–5000-fold in K_i values. As in the case of the amides, the introduction of polar groups in the side chain provides some damping effect on inhibition by hydroxamic acids.

Table II: Inhibition of *Aeromonas* Aminopeptidase by Hydroxamates[a] and Related Compounds

inhibitor	K_i (M)
hydroxylamine	2.2×10^{-1}
formyl hydroxamate	3.0×10^{-4}
acetohydroxamate	2.0×10^{-4}
glycine hydroxamate	7.7×10^{-5}
L-alanine hydroxamate	2.0×10^{-5}
L-threonine hydroxamate	6.6×10^{-5}
L-valine hydroxamate	2.2×10^{-6}
L-leucine hydroxamate	3.5×10^{-7}
L-isoleucine hydroxamate	2.0×10^{-6}
L-phenylalanine hydroxamate	8.0×10^{-6}
L-tyrosine hydroxamate	8.3×10^{-5}
L-histidine hydroxamate	7.8×10^{-5}
L-lysine hydroxamate	5.5×10^{-5}
L-tryptophan hydroxamate	2.2×10^{-4}

[a] No hydrolysis of any hydroxamate was detected.

Interactions between Inhibitors. We have previously reported that BuBA is an excellent inhibitor of *Aeromonas* aminopeptidase, with a K_i value of 9.6×10^{-6} M (Baker & Prescott, 1980). With the finding that hydroxamic acids are powerful inhibitors of other classes of metallopeptidases and with the demonstrated involvement of zinc in the binding of hydroxamic acids by thermolysin (Nishino & Powers, 1978; Holmes & Matthews, 1981), it seemed important to evaluate the possibility that these two types of inhibitors bind to the same locus. The kinetic analysis of Yonetani & Theorell (1964) was therefore applied in order to determine whether the binding of BuBA was mutually exclusive with binding of L-leucine hydroxamate. Similar tests were made for the inhibitor pairs BuBA and formyl hydroxamate and for L-leucine hydroxamate and formyl hydroxamate. The results shown in Figure 1 reveal that each of these three inhibitors showed mutual exclusivity with the other two over a range of $5K_i$. A different type of interaction between two inhibitors and the enzyme is shown in Figure 2A in which a hydrophobic compound, isoamyl alcohol, is paired with formyl hydroxamate, resulting in simultaneous binding of both inhibitors. The observed interaction is intermediate between the complete mutual exclusivity shown for the three inhibitors in Figure 1 and the case in which binding of one inhibitor is without effect

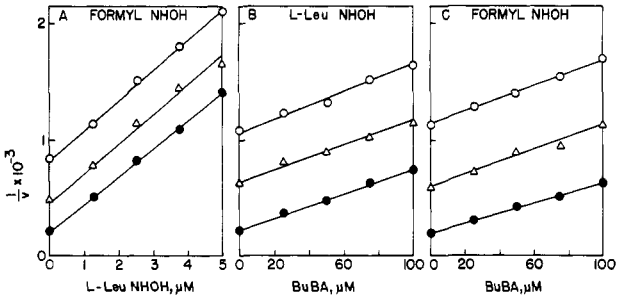

FIGURE 1: Multiple inhibition of *Aeromonas* aminopeptidase by (A) L-leucine hydroxamate in the presence of (●) 0, (△) 0.99, and (○) 1.97 mM formyl hydroxamate, (B) BuBA in the presence of (●) 0, (△) 2.5, and (○) 5.0 μM L-leucine hydroxamate, and (C) BuBA in the presence of (●) 0, (△) 0.99, and (○) 1.97 mM formyl hydroxamate.

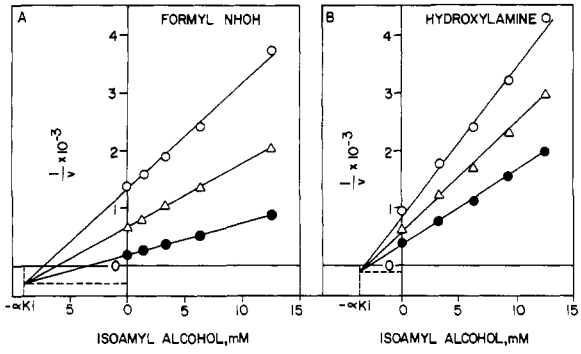

FIGURE 2: Multiple inhibition of *Aeromonas* aminopeptidase by (A) isoamyl alcohol and (●) 0, (△) 1.5, and (○) 3.0 mM formyl hydroxamate and (B) isoamyl alcohol and (●) 0, (△) 0.5, and (○) 1.0 M hydroxylamine.

on the binding of the other; Figure 2B shows that even less interference in binding occurs between isoamyl alcohol and hydroxylamine. Increasing the size of the hydroxamate from formyl hydroxamate to acetyl hydroxamate, however, resulted in complete mutual exclusivity between hydroxamate and isoamyl alcohol.

Discussion

The incorporation of metal-coordinating groups into substrate-analogue inhibitors has resulted in compounds that are potential mechanistic probes for metalloproteases. Although Holmquist & Vallee (1979) cautioned that not all details of inhibitor interactions can be applied to the binding of substrates, these workers nevertheless have been successful in establishing the ligand geometry of inhibitors of carboxypeptidase A. The metal-binding ligands that apparently are most effective with carboxypeptidase A are the anionic forms of mercapto or phosphoryl groups (Kam et al., 1979; Holmquist & Vallee, 1979). For another zinc metalloprotease, angiotensin converting enzyme, the efficiency of the mercapto group is shown by the K_i value of 1.7×10^{-9} M for (D-3-mercapto-2-methylpropanoyl)-L-proline (Captopril) (Cushman et al., 1977). Of the wide array of potential metal-binding inhibitors that have been tested, the most effective ones for thermolysin and *Pseudomonas aeruginosa* elastase were those with a hydroxamido moiety, present either as a free hydroxamic acid group (-CONOH) or as a portion of an N^{α}-acyl-N^{α}-hydroxy peptide (Nishino & Powers, 1978, 1979, 1980). These investigators showed that substitution of a methyl group for either of the two hydrogens of the NHOH group drastically reduced binding of the inhibitors. This suggested that bidentate complexes were formed between the inhibitor and the active site zinc, a hypothesis that was later confirmed in the X-ray crystal structure study of thermolysin–hydroxamido complexes by Holmes & Matthews (1981).

From these results with other metalloproteases and the results reported herein for *Aeromonas* aminopeptidase, it follows that the most likely mechanism for the inhibition of *Aeromonas* aminopeptidase by hydroxamates involves their binding to the active site zinc. This is substantiated by the fact that formyl hydroxamate—a compound capable of forming bidentate ligands with zinc—is 4000-fold more effective as an inhibitor than formamide, its deoxy analogue. Similarly, the hydroxamates of glycine and alanine are 3 orders of magnitude more tightly bound than the corresponding amides. That inhibition by hydroxamates is caused by the formation of a ternary complex, rather than by removal of zinc from the enzyme, was shown by experiments in which the concentration of zinc ions in the reaction was varied, without effect on inhibition. With both amino acid amides and hydroxamates, binding was enhanced by bulky, hydrophobic side chains; e.g., the hydroxamates of valine, leucine, isoleucine, and phenylalanine produced 10–100-fold greater inhibition than alanine hydroxamate. For those compounds with side chains of equivalent size and shape, binding to *Aeromonas* aminopeptidase was strongly influenced by the polarity of the side chain (cf. threonine and valine and tyrosine and phenylalanine).[2] Inasmuch as there is a distinct parallel between the relative order of binding for a series of amino acid amides (which are substrates for *Aeromonas* aminopeptidase) and their analogous hydroxamates, we conclude that the hydroxamate inhibitors are bound with their side chains in the same orientation as the substrates.

A major objective of the investigation was to determine the position of the essential zinc atom in relation to other logically necessary features of the active site. In order to obtain evidence for the proximity of the respective binding loci, therefore, we carried out the multiple-inhibition studies in which hydroxamates were used as reagents for the complexing of enzyme-bound zinc, BuBA was the marker for the catalytic subsite, and isoamyl alcohol was utilized as a marker for the hydrophobic site. Yonetani & Theorell (1964) have presented a general steady-state kinetic method for determining the extent of interference between two inhibitors binding to the same enzyme. In this treatment, reciprocal velocities at one substrate concentration in the range of K_m are measured in the presence of a range of concentrations of one inhibitor (I_1) at each of several different concentrations of a second inhibitor (I_2), the reciprocal velocities being plotted against concentration of I_1 as abscissa. Complete mutual exclusivity of binding, in which the binding of one inhibitor prevents the binding of the other, yields families of parallel lines for the different fixed concentrations of the second inhibitor. Conversely, independent binding, in which the binding of one inhibitor has no effect on the binding of the other, results in families of stright lines, intersecting to the left of the $1/v$ axis at a concentration of I_1 equal to K_i for this inhibitor. The extent to which a given system of an enzyme and two inhibitors (I_1 and I_2) resembles either of these "ideal" cases is conveniently expressed with the interaction constant α as shown in

[2] During the preparation of the manuscript, Chan et al. (1982) reported inhibition of the microsomal leucine aminopeptidase from porcine kidney by several hydroxamates, but both the absolute and relative magnitudes of K_i values they observed differed considerably from those of *Aeromonas* aminopeptidase. With the kidney enzyme, there was little difference in the K_i values for the hydroxamates of L-leucine, L-tyrosine, L-lysine, and DL-valine; in contrast, the *Aeromonas* enzyme showed a high degree of specificity for hydroxamates of the branched-chain amino acids.

eq 1 (Yonetani & Theorell, 1964):

$$\text{(slope with } I_2)/\text{(slope without } I_2) = 1 + [I_2]/(\alpha K_{EI_2}) \quad (1)$$

in which the slopes referred to are the slopes of plots of reciprocal velocity vs. $[I_1]$ at a fixed concentration of substrate. For complete mutual exclusivity (parallel lines), the value of α is infinite; for pairs of inhibitors capable of binding simultaneously (intersecting lines), $0 < \alpha < \infty$. For these intersecting patterns, the point of interaction is at an abscissa value equal to $-\alpha K_{i1}$, where K_{i1} is the K_i for I_1, measured separately. Values of α between 1.0 and infinity are measures of the factor by which the binding of one inhibitor to the enzyme decreases the affinity of the enzyme for the other inhibitor. Values of α between 1.0 and 0, on the other hand, indicate positive cooperativiity between binding of the two inhibitors. The kinetic results shown in Figure 1 demonstrate that binding of any one of three competitive inhibitors—BuBA, L-leucine hydroxamate, or formyl hydroxamate—prevents binding of either of the other two, indicating mutual exclusivity. The existence of a hydrophobic side chain on both BuBA and L-leucine hydroxamate raised the possibility that the mutual exclusivity between them may be partially attributable to competition for a hydrophobic pocket, in addition to competition between the dihydroxyboron and hydroxamido moieties. The competition between formyl hydroxamate and each of the other two inhibitors (Figure 1B,C), however, involves no such ambiguity in identification of competing groups, inasmuch as formyl hydroxamate, not having a hydrophobic side chain, can compete only with the polar portions of L-leucine hydroxamate and BuBA. The fact that formyl hydroxamate prevents the binding of BuBA, therefore, shows that the active site zinc (hydroxamido binding site) and the locus of binding for BuBA overlap. On the basis of the transition state analogue nature of the boronic acid inhibition of *Aeromonas* aminopeptidase (Baker & Prescott, 1980) and of a variety of serine proteases (Koehler & Lienhard, 1971; Philipp & Bender, 1971; Matthews et al., 1975), one would expect the dihydroxyboron group to be bound to the catalytic subsite; it then follows that there is either identity or close proximity between the active site zinc and one of the enzyme groups of the catalytic subsite.

In contrast to the mutual exclusivity shown by these three pairs of inhibitors (showing overlapping binding sites), mutual exclusivity would not be expected from pairs of inhibitors that bear one but not both requisite segments of the preferred substrates: i.e., the hydrophobic side chain and the carboxamido group. This possibility was tested by using isoamyl alcohol, a competitive inhibitor of *Aeromonas* aminopeptidase, to represent the hydrophobic moiety and formyl hydroxamate to serve as a polar binding reagent. The former contains a hydrophobic side chain with only an alcohol functional group; the latter is devoid of a side chain. The intersecting lines in Figure 2A show that the active site of *Aeromonas* aminopeptidase is capable of accommodating both isoamyl alcohol and formyl hydroxamate. The α value of 7.5 indicates, however, that some interference with simultaneous binding exists. This is not unexpected, as the carboxamido group of aminopeptidase substrates occupies approximately 4 Å and formyl hydroxamate about 5–6 Å. It thus seems clear that the hydroxamido group in the inhibitors binds to the locus in the enzyme that is occupied by the carboxamido group of substrates. Decreasing the size of the polar inhibitor by use of hydroxylamine (4 Å × 5 Å × 2.8 Å) in conjunction with isoamyl alcohol resulted in an α value of 2 (Figure 2B), but increasing the size by use of acetohydroxamate completely excluded binding of isoamyl alcohol (Figure 3). A comparison

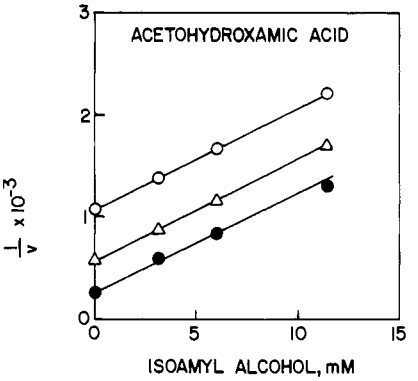

FIGURE 3: Multiple inhibition of *Aeromonas* aminopeptidase by isoamyl alcohol and (●) 0, (△) 0.4, and (○) 1.0 mM acetohydroxamic acid.

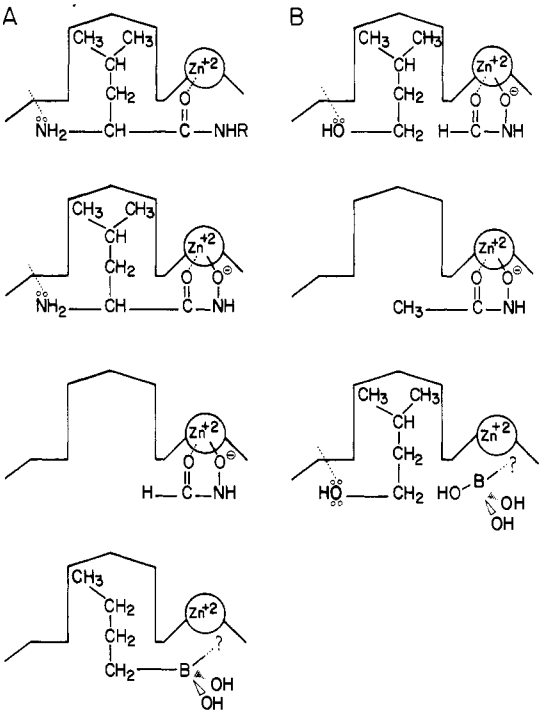

FIGURE 4: Schematic representation of binding of substrates and inhibitors to *Aeromonas* aminopeptidase. (A) Proposed analogies between the binding of substrates and hydroxamate and boronic acid inhibitors. All inhibitors shown in (A) are proposed to bind in the carboxamido binding site. (B) Binding modes proposed to explain the observations that formyl hydroxamate and boric acid were each bound to the aminopeptidase simultaneously with binding of isoamyl alcohol while binding of acetohydroxamate and binding of isoamyl alcohol were mutually exclusive.

of the results with formyl hydroxamate and acetohydroxamate reveals the pronounced effect exerted by a methylene group in crowding the hydrophobic pocket.

Additional evidence for the loci of binding was obtained from experiments using boric acid, a weak inhibitor (K_i = 0.13 M) of *Aeromonas* aminopeptidase. BuBA was excluded from the active site by the presence of borate; parallel lines similar to those in Figure 1 were evidence for the specific binding of the dihydroxyboron group at a single site. When isoamyl alcohol inhibition was tested in the presence of boric acid (0.3 and 0.6 M), however, a pattern of intersecting lines was obtained, and the interaction constant (α = 2.4) showed that little interference occurred between the hydrophobic group of isoamyl alcohol and the dihydroxyboron group. Figure 4 shows the structures of the inhibitors with a schematic depicting the analogy between alignment of substrates and inhibitors in the

enzyme active site. This schematic does not presuppose the identity of the nucleophile that adds to the bound boric acid or BuBA (hence, the question mark in Figure 4). The inhibitors depicted in Figure 4A exhibited mutually exclusive binding as did acetohydroxamate and isoamyl alcohol depicted in Figure 4B; the alignment of isoamyl alcohol with boric acid and formyl hydroxamate to permit simultaneous binding is also shown. Some other aminopeptidases possess, in common with *Aeromonas* aminopeptidase, an essential zinc ion and show a preference for bulky hydrophobic groups. We have found cytosolic leucine aminopeptidase from porcine kidney to be effectively inhibited both by boronic acid inhibitors and by compounds having a hydroxamido group. Hydrolysis of L-leucine *p*-nitroanilide by manganese-acetivated cytosolic leucine aminopeptidase (Sigma Type III CP, lot 111F-8015) was inhibited by formyl hydroxamate ($K_i = 1.9 \times 10^{-4}$ M), L-alanine hydroxamate ($K_i = 2.0 \times 10^{-5}$ M), and L-leucine hydroxamate ($K_i = 8 \times 10^{-6}$ M).

Possible Roles for Zinc. Although the mechanism of peptide hydrolysis is not known for any aminopeptidase, analogy with nonenzymatic hydrolyses implies the logical necessity of at least three enzyme functional groups that interact directly with the scissile bond of substrate: a nucleophile that attacks the carbonyl carbon, an electron sink that accommodates that negatively charged oxygen of the resulting oxyanion intermediate, and a group that protonates the leaving-group nitrogen during breakdown of the intermediate to form products.

Chemical modification studies have shown that at least one tyrosine residue and one carboxyl group are essential for catalysis by *Aeromonas* aminopeptidase, and these residues should be considered in assigning the catalytic roles described above; histidine and tryptophan residues have been shown to be nonessential (Baker & Prescott, 1980; Mäkinen et al., 1982a,b). Because of the amphoteric nature of zinc, it is at least theoretically possible that the essential zinc ion of *Aeromonas* aminopeptidase could fulfill any one of the essential catalytic roles, through nucleophilic attack by a zinc-bound hydroxyl ion (Woolley, 1975), through stabilization of the oxyanion by the zinc ion, acting as an electron sink (Vallee et al., 1963; Fersht, 1977), or through donation of a proton by a water molecule made far more acidic than ordinary water by coordination to the metal ion (Dworschack & Plapp, 1977). While the results presented in this paper cannot be used to assign a specific catalytic role to the zinc in the enzyme, the demonstrated close proximity of the bound zinc to the position of the logically essential catalytic groups strongly suggests that the bound metal ion is one of the enzyme functional groups interacting directly with the scissile bond during catalysis.

Registry No. BuBA, 4426-47-5; EC 3.4.11.10, 37288-67-8; formyl hydroxamate, 4312-87-2; ethyl chloroformate, 541-41-3; hydroxylamine hydrochloride, 5470-11-1; L-alanine hydroxamate, 2508-25-0; L-isoleucine hydroxamate, 31982-77-1; L-phenylalanine hydroxamate, 19254-08-1; Boc-L-phenylalanine *N*-hydroxysuccinimide ester, 3674-06-4; hydroxylamine, 7803-49-8; acetohydroxamate, 546-88-3; glycine hydroxamate, 5349-80-4; L-threonine hydroxamate, 84926-49-8; L-valine hydroxamate, 28664-96-2; L-leucine hydroxamate, 31982-78-2; L-tyrosine hydroxamate, 4985-42-6; L-histidine hydroxamate, 25486-00-4; L-lysine hydroxamate, 25125-92-2; L-tryptophan hydroxamate, 14954-40-6; formamide, 75-12-7; glycine amide, 598-41-4; L-alanine amide, 7324-05-2; L-valine amide, 4540-60-7; L-threonine amide, 49705-99-9; L-methionine amide, 4510-08-1; L-leucine amide, 687-51-4; L-isoleucine amide, 14445-54-6; L-norleucine amide, 7324-07-4; L-phenylalanine amide, 5241-58-7; L-tyrosine amide, 4985-46-0; L-tryptophan amide, 20696-57-5; L-valine, 72-18-4; L-leucine, 61-90-5; L-isoleucine, 73-32-5; boric acid, 10043-35-3; methylboronic acid, 13061-96-6; phenylboronic acid, 98-80-6; isoamyl alcohol, 123-51-3.

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