Aeromonas Aminopeptidase: pH Dependence and a Transition-State-Analogue Inhibitor†

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ABSTRACT: The mechanism of the hydrolysis of L-leucine-*p*-nitroanilide by *Aeromonas* aminopeptidase has been investigated by a combination of pH dependence and competitive inhibition experiments. The pH dependence of the buffer-independent value of K_m indicates that binding of the substrate to the enzyme requires the free-base form of a group (apparently the substrate α-amino group) ionizing near pH 7.6 and the undissociated form of two enzyme groups ionizing between pH 10 and pH 11. The pH dependence of the catalytic rate constant, k_{cat}, indicates that the free-base form of an enzyme functional group with pK_a near 5.3 is required for hydrolysis of bound substrate. 1-Butaneboronic acid (BuBA) is a potent inhibitor of the aminopeptidase ($K_i = 9.6 \times 10^{-6}$ M at pH 8.0), binding more than 200 times as tightly as the essentially isosteric and electronically similar substrate analogue *n*-valeramide ($K_i = 2.16 \times 10^{-3}$ M at pH 8.0). Inhibition by BuBA decreases at low pH, as does the catalytic rate constant, whereas the inhibition by *n*-valeramide shows no such decrease. These results suggest that the boronic acid combines with the enzyme to form an analogue of the transition state for hydrolysis of substrate. Such interaction between BuBA and *Aeromonas* aminopeptidase, a zinc metalloenzyme that has been shown not to have an essential serine residue [Prescott, J. M., & Wilkes, S. H. (1966) *Arch. Biochem. Biophys. 117*, 328–336], indicates that transition-state-analogue inhibition by boronic acids may be more general with regard to acylhydrolases than previously supposed.

Of the three major classifications of peptidohydrolytic enzymes, the carboxypeptidases and the endopeptidases have been the subjects of detailed mechanistic studies. In both of these two categories, our understanding of the general mechanistic features of the class of enzymes as a whole has been greatly advanced by work concentrated on one member of the group—α-chymotrypsin in one case and carboxypeptidase A in the other. Considerably less attention has been focused on the aminopeptidases as a group, and thus far no member of the group has been singled out for the intensity of study accorded chymotrypsin and carboxypeptidase A. *Aeromonas* aminopeptidase (EC 3.4.11.10) is an excellent subject for studies aimed at a general understanding of aminopeptidase mechanisms. This enzyme, a zinc metalloprotein of M_r 29 500 (Prescott & Wilkes, 1966; Prescott et al., 1971), is secreted into the culture medium of the marine bacterium *Aeromonas proteolytica* (ATCC 15338), from which it can be isolated in gram quantities, in a highly purified form devoid of endopeptidase activity (Prescott et al., 1971). The high degree of stability of the enzyme makes it attractive for extended kinetic studies, and because *Aeromonas* aminopeptidase is monomeric, the interpretation of kinetic data can be expected to be considerably simpler than in the case of the multimeric mammalian aminopeptidases. Detailed information exists concerning the physical properties of the aminopeptidase (Prescott et al., 1971), its specificity toward amide, dipeptide, and oligopeptide substrates (Wagner et al., 1972; Wilkes et al., 1973), and its inhibition by leucine methyl ketone substrate analogues (Kettner et al., 1974). The hydrolysis of L-leucine-*p*-nitroanilide (LPNA)[1] by *Aeromonas* aminopeptidase provided a convenient spectrophotometric assay, the high sensitivity of which is essentially independent of pH above 3.0 (Tuppy et al., 1962). The availability of an isolation procedure (see above) capable of providing gram quantity batches of the enzyme makes it an attractive subject for chemical modification studies.

Studies of the pH dependence of an enzymic reaction can identify kinetically significant enzyme and substrate ionizing groups in terms of their apparent pK_a values and possible functions in the reaction. In conjunction with chemical modification studies, these kinetically detected ionizations can be used to make tentative identifications of the specific amino acid residues involved. As an integral part of our active site mechanistic studies of *Aeromonas* aminopeptidase—from which we have already reported the results of chemical modification (Baker & Prescott, 1980; Mäkinen et al., 1982a,b) and marker inhibitor experiments (Baker et al., 1983)—we therefore have been engaged in an investigation of the pH dependence of the hydrolysis of LPNA by this enzyme.

During a preliminary series of buffer-screening experiments, undertaken to ensure that the K_m and k_{cat} values obtained were intrinsic to the enzyme–substrate system and not buffer-dependent artifacts, we noted the sharply pH-dependent inhibition of the enzyme reaction by boric acid buffers. Experiments following up on this observation led to the finding that 1-butaneboronic acid (BuBA) exhibits the properties of a transition-state-analogue inhibitor.

Experimental Procedures

Enzyme. Aeromonas aminopeptidase, isolated from the culture filtrate of *Aeromonas proteolytica* by the procedure of Prescott & Wilkes (1976), was kindly provided by S. H. Wilkes. The enzyme was stored in the frozen state until

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[1] Abbreviations: BuBA, 1-butaneboronic acid; CAPS, (cyclohexylamino)propanesulfonic acid; LPNA, L-leucine-*p*-nitroanilide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Taps, [[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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needed for the experiments, whereupon it was thawed and diluted in 1 mM Tricine buffer, pH 8.0, that contained 0.2 M KCl and 0.1 mM $ZnSO_4$.

Enzyme Assays. Aminopeptidase activity against LPNA was measured spectrophotometrically by the increase in absorbance at 404 nm (Tuppy et al., 1962) in cells with a 4-cm light path. Absorbance changes were recorded in a Zeiss PMQ-II spectrophotometer, the sample compartment of which was maintained at 25 °C. Enzyme dilutions to be used in determination of K_m, k_{cat}, and K_i values for profile studies were maintained at pH 8.0 in 1.0 mM Tricine containing 0.2 M KCl and 1.0×10^{-4} M $ZnSO_4$; in experiments designed to detect any effect of varying the zinc concentration in the assay mixtures at high pH, the (pH 8.0) enzyme dilutions were 2.0×10^{-6} M in zinc. Assays were initiated by adding 0.05 mL of the enzyme dilution (pH 8.0) to 10 mL of substrate solution buffered at the pH of the particular experiment. Buffers selected for use in each pH range were either those that exhibited negligible inhibition of the reaction or, in the event that no completely noninhibitory buffer was found for use in a given pH range, the buffer of choice was that which was found least inhibitory among those exhibiting linear competitive inhibition (see below). For these buffers, values of K_m and k_{cat} were determined at several concentrations of buffer, and the values were extrapolated to zero buffer concentration in order to obtain the buffer-independent values. At each concentration of buffer, the initial rate of hydrolysis was determined in triplicate at each of five to seven different substrate concentrations in the range 0.2–5.0 K_m, the ionic strength being held constant at 0.2 by addition of KCl. Values of K_m and k_{cat} were determined by least-squares linear regression analysis of the double-reciprocal plots by using the prerecorded programs STAT 105A and STAT 1-22A (Stat Pac 1, Hewlett Packard Co.) for the Hewlett Packard HP-65 calculator. Theoretical pH dependence curves shown in the figures are visual best fits generated with the aid of a plotting routine written in PASCAL for the Apple II Plus computer.

No explicit "buffer corrections" were applied to the K_i values determined. Instead, at each pH that buffer was selected that could control the pH adequately at the concentration equal to less than 0.1 (and in most cases, less than 0.05) times the value of K_i for inhibition by the buffer. On the basis of preliminary experiments, inhibitor concentrations were selected at each pH to equal approximately 1–4 times the value of K_i for a given inhibitor. The value of K_i was then determined from a replot vs. inhibitor concentration of the slopes of the $1/S$ vs. $1/v$ plots.

Zinc Concentrations in Assay Mixtures. In order to prevent the pH profile from being overlaid by artifacts stemming from the loss of zinc from the enzyme, experiments were so designed that loss of zinc was prevented by additions of Zn^{2+} ions according to the pH value of the assay and according to the abilities of the different buffers to sequester metal ions. Below pH 6.5 where dissociation of zinc was potentially troublesome, K_m and V_{max} were determined in from three to six different concentrations of zinc at each pH, and the values obtained were extrapolated, by using double-reciprocal plots of $1/K_m$ and $1/V_{max}$ against $1/[Zn]$, to those K_m and V_{max} values that would have resulted were there no loss of zinc. The assays at low pH values were run by allowing enzyme, added zinc, and substrate to equilibrate together briefly before the slopes of the tracings were measured. Enzyme concentrations were selected so that no more than 5% of the substrate was hydrolyzed during the equilibration and assay periods. In the range from pH 7.0 to 8.5 (Tricine buffer), sufficient zinc was added to make the assay mixtures 1×10^{-4} M in zinc. In the range above pH 8.5 (borate and carbonate buffers), no zinc was added to the reaction mixture other than that contained in the 0.05 mL of enzyme solution. The total concentration of zinc added to these alkaline-range assay mixtures was therefore 5.0×10^{-7} M at maximum.

Determination of Acid Dissociation Constants. Titrations were carried out at 25 °C in 0.2 M KCl. Dissolved CO_2 was removed from the samples by purging for 30 min with water-saturated nitrogen, prior to titration; the samples were maintained under a nitrogen atmosphere during titration with KOH. Corrections were made for dilution of sample by addition of titrant, and for titration of solvent. In the titration of the α-amino group of L-leucine-*p*-nitroanilide (1.0 mM, initial volume 50 mL), the titrant was 0.0894 M KOH, standardized against potassium hydrogen phthalate; concentrations of protonated and free-base LPNA were calculated from the known initial concentration of LPNA (added as the hydrochloride) and the number of moles of KOH added, and pK_a was determined from plots of log $[A/AH^+]$ vs. pH. In the titration of 1-butaneboronic acid (BuBA), the ability of D-mannitol to increase the apparent acidity of boric and boronic acids (Davis & Mott, 1980; Mikan & Bartusek, 1980) was employed to circumvent the necessity for the large solvent-titration corrections required in determination of end points above pH 11. First, the end-point equivalence of the sample of BuBA (50 mM, 8 mL) was determined in terms of the KOH titrant used (nominal 0.5 M) by titration of the sample in the presence of 0.5 M mannitol. An otherwise identical sample, without mannitol, was then titrated to the half-equivalence point. After minor correction of the titrant volume for titration of solvent, the pK_a was calculated from the resulting pH and the exact equivalence of the volume of KOH titrant added.

Other Methods. Stock solutions of substrates and buffers used to study the effects of low concentrations of zinc were extracted with dithizone (Chemical Dynamics Corp.) in carbon tetrachloride (Fisher certified 99 mol % pure). For buffer–salt solutions the metal extraction was carried out with the aqueous phase adjusted to pH 8.3 (Sandell, 1959) by using three successive portions (each one-tenth the volume of the aqueous phase) of dithizone solution (0.25 mg/100 mL), followed by five extractions with CCl_4 (aqueous phase at pH 6.8) to remove excess dithizone. Because of the solubility of unprotonated LPNA in CCl_4, extraction of the 10 mM substrate stock was carried out with the aqueous phase at pH 5.5, with a more concentrated solution of dithizone (10 mg/100 mL) being used to compensate for the decreased partitioning of dithizone into the aqueous phase at the lower pH. Removal of excess dithizone was accomplished by five CCl_4 extractions at pH 4.5. Recovery of the substrate, as measured spectrophotometrically at 315 nm (Tuppy et al., 1962) was better than 94%. The extracted stock solutions were adjusted to the appropriate pH with KOH or HCl and made up to volume, either with "Milli-Q"[2] water (16–18 MΩ cm and less than 4.0×10^{-9} M Zn) or with glass-distilled water having less than 2.0×10^{-9} M Zn, to yield working solutions approximately 5.0×10^{-8} M in zinc. Zinc concentrations of the final substrate and buffer solutions were determined with a Perkin-Elmer Model 272 atomic absorption spectrophotometer equipped with graphite furnace and deuterium-arc background corrector by using internal standards (Method of Additions) to determine

[2] "Milli-Q" is a registered trademark of the Millipore Corp., Bedford, MA.

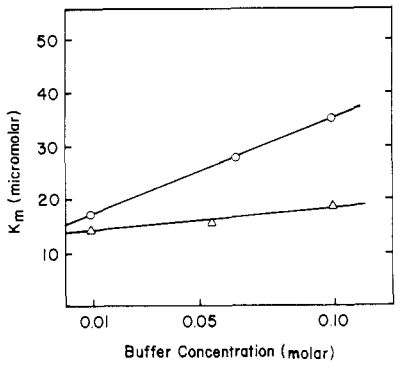

FIGURE 1: Extrapolation of K_m values to zero buffer concentration to obtain buffer-independent values. Results shown are those obtained at pH 8.5 with boric acid/KOH (○) and Tricine/KOH (△) buffer systems.

sensitivity under our conditions. When zinc was added to any solutions of buffer or enzyme, Fisher SO-Z-12 atomic absorption standard, Johnson-Matthey Specpure $ZnSO_4$, or Johnson-Matthey Specpure ZnO dissolved in HCl was used.

Results

Effects of Buffers on Activity. Six buffers, all of which showed linear competitive inhibition, were found satisfactory for investigation of the effects of pH on kinetic parameters. Acetate (pH 4.7–5.5), Mes (pH 5.5–6.5), and Mops (pH 6.5–7.0) all exhibited K_i values greater than 0.5 M in the indicated pH ranges and had more than adequate buffering capacity at a concentration of 10 mM. Tricine had K_i values greater than 0.35 M in its range of use (pH 7.0–8.5). The inhibition by boric acid/borate buffer is sharply pH dependent over the range from pH 8.0 to 10.5, but above pH 8.5 the value of K_i is 0.1 M or larger, allowing the use of borate as primary buffer between pH 8.5 and 10. From pH 10.0 to 10.7, carbonate buffer exhibited K_i^m values greater than 0.3 M. The buffers found to be unsuitable were the following: phosphate, which tends to remove zinc from the solution; Tris and Taps, which cover roughly the same pH range as Tricine but were considerably more inhibitory; CAPS (pK_a = 10.4), which not only was highly inhibitory but also exhibited parabolic, rather than linear, competitive inhibition. Extrapolation of K_m values obtained in various buffers to zero buffer concentration gave acceptable agreement between buffers, as is illustrated by Figure 1, which compares the results obtained with borate and Tricine buffer systems at the boundary between their respective pH ranges.

Effects of Zinc Concentration. Our previous work has shown *Aeromonas* aminopeptidase to be a metalloenzyme that is inactivated by the loss of bound zinc (Prescott & Wilkes, 1966, 1976). In the present experiments, it was essential that the enzyme retain its full content of zinc at all pH values and with all buffers that were used, in order to prevent artifacts due to the loss of zinc from the holoenzyme. Metals dissociate from metalloenzymes more readily in the lower pH ranges than in the higher; moreover, different buffers possess varying abilities to form complexes with metal ions. Accordingly, the effect of varying the zinc concentration in the assay was studied in each of the pH ranges covered. For the range of conditions used in this investigation, the only significant effects exerted by varying the concentration of zinc in the assay mixtures occurred at pH values below 6.5, these effects being due to the dissociation of bound metal from the enzyme. At pH 6.5 and below, therefore, it was necessary to correct for the dissociation of zinc from the enzyme by determining K_m and V_{max} at several concentrations of zinc at each pH value and then extrapolating, by means of double-reciprocal plots of $1/K_m$ and $1/V_{max}$ vs. $1/[Zn]$, to the values that would have resulted had there been no dissociation of zinc. The results of these experiments are shown in Figure 2.[3] Representative primary data (tracings of absorbance vs. time at different levels of zinc) are shown in Figure 2A. During equilibration of enzyme, zinc, and substrate the rate of the hydrolysis decreased smoothly from its initial value to a constant "equilibrated" value characteristic of that particular combination of zinc and substrate concentrations (Figure 2A); the slope was then taken in the linear portion immediately following the initial, nonlinear equilibration period. In the plots of both reciprocal k_{cat} (Figure 2B) and normalized reciprocal K_m (Figure 2C) the slopes of the plots decrease rapidly as the pH is increased from 5.0 to 6.5, with the enzyme activity becoming essentially independent of zinc concentration at pH 6.5 and above. Our addition of zinc to the low pH assays to prevent depletion of the enzyme-bound zinc is similar to the approach used by Auld & Vallee (1970), in their study of the pH dependence of zinc carboxypeptidase A. These workers used excess zinc to suppress the dissociation of the catalytically essential zinc of the carboxypeptidase and noted, as we have in the case of *Aeromonas* aminopeptidase, that the effect of excess zinc on enzyme activity decreased rapidly as the pH was increased from 4.8 to 6.2.

In the pH range 7.0–8.5, Tricine buffer is known to sequester Zn^{2+} ions (Vieles et al., 1972), but the effect on the aminopeptidase reaction was negligible except at Tricine concentrations close to 100 mM and was easily prevented by rendering the assays in this pH range 1×10^{-4} M in $ZnSO_4$. At higher pH values (8.5–10.5), complexation of zinc by hydroxide ions and the limited solubility of $Zn(OH)_2$ sharply restrict the range of possible concentrations of both total zinc and free Zn^{2+} ions (Latimer, 1952; Perrin, 1969). At pH 10.0, in dithizone-extracted boric acid–KOH buffer, varying the total zinc in the assay from 5.8×10^{-8} to 5.5×10^{-7} M produced no effect on either K_m or k_{cat}. In no part of the pH range studied, therefore, were the observed effects of pH on K_m and k_{cat} due to depletion of bound zinc, and in no part of the range studied was inhibition by zinc a factor in the results.

pH Dependence of k_{cat}, K_m, and K_i. The pH dependence of pK_m (Figure 3A) indicates that binding of substrate to enzyme requires the free-base form of a group ionizing in either free enzyme or free substrate with a pK_a near 7.6 and also requires the acidic, undissociated, forms of more than one enzyme group ionizing between pH 10 and pH 11. The small wave between pH 4 and pH 6 in the plot of pK_m vs. pH was consistently observed. The plot of log k_{cat} as a function of pH is simpler than that of K_m, showing a single inflection near pH 5.3 (Figure 3C)—evidently a reflection of the need for an ionizing group with pK_a near 5.3 in the ES complex to be in the free-base form in order for bound substrate to be hydrolyzed. Due to the fact that k_{cat} is independent of pH between 8.5 and 10.7, the pH dependence of log k_{cat}/K_m (Figure 3B) in this region is identical with that of pK_m. Between pH 8.5 and pH 6.0, the contribution from the k_{cat} curve is minor, and the k_{cat}/K_m curve is dominated by the pH dependence of pK_m.

[3] It has been determined, through experiments designed to measure displacement of protons from the substrate amino group upon titration with zinc ion, that the substrate LPNA and zinc ion are not appreciably complexed in the pH range of the experiments illustrated in Figure 2. When 20 mM LPNA, self-buffered at pH 5.5, was titrated to a concentration of 10 mM in zinc ions, there was no observable decrease in pH. Under the conditions of this experiment, complexation of as little as 1% of the substrate would have been readily detected.

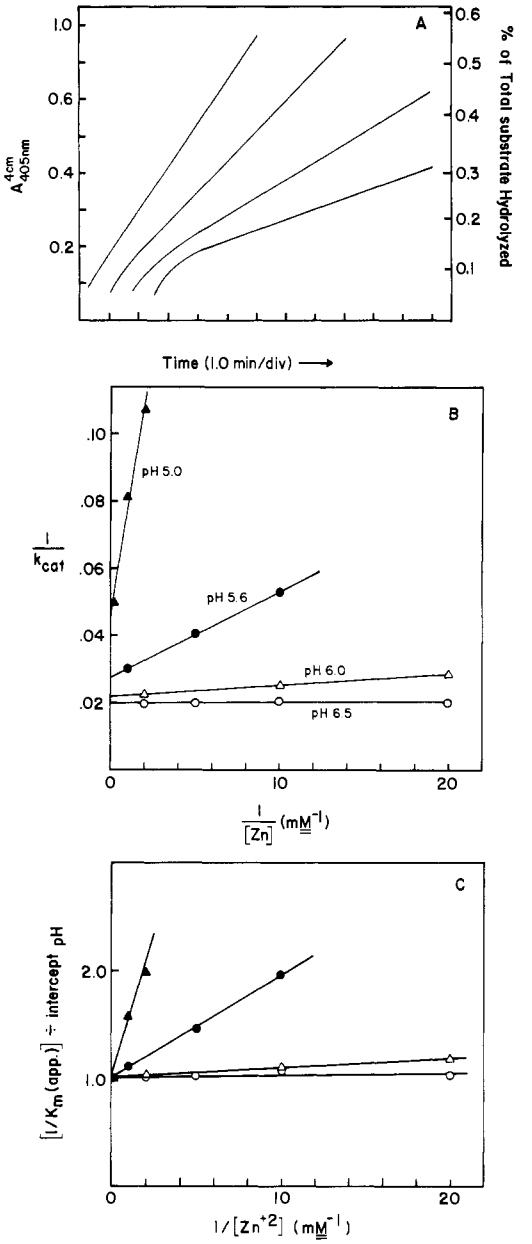

FIGURE 2: Extrapolation of k_{cat} and K_m values to saturating Zn^{2+} concentrations to obtain values for that form of the enzyme having a full complement of bound Zn^{2+}. (A) Primary data, pH 5.0, showing progress curves (recorder tracings) for hydrolysis of LPNA by *Aeromonas* aminopeptidase at a constant level of substrate (4 mM) and four different levels of Zn^{2+} (from left to right: 1.0, 0.5, 0.2, and 0.1 mM Zn^{2+}). (B) Reciprocal $k_{cat(obsd)}$ plotted as a function of the reciprocal of the Zn^{2+} concentration in the assay: (○) pH 6.5; (△) pH 6.0; (●) pH 5.6; (▲) pH 5.0. (C) Reciprocal $K_{m(obsd)}$ plotted as a function of the reciprocal of the Zn^{2+} concentration in the assay: (○) pH 6.5; (△) pH 6.0; (●) pH 5.6; (▲) pH 5.0. In order to avoid difficulties of scale (since the actual values of the intercepts vary by a factor of more than 16 over the pH range shown), and in order to emphasize the effect of pH on the slopes of the plots, the values plotted for a given pH are the values of $K_{m(obsd)}$ at that pH, divided by the intercept on the $1/K_{m(obsd)}$ axis for that pH ($intercept_{pH}$).

Considerably more interaction between the pH dependences of K_m and k_{cat} is shown in the region below pH 6.0. Whereas k_{cat} shows a single inflection at pH 5.3 and K_m shows a wave with inflections at pH 5.3 and 4.8, the plot of log k_{cat}/K_m has a single inflection near pH 4.8.

Figure 3D illustrates the pH dependence of K_i for several inhibitors of the hydrolysis of LPNA. All of the inhibitors shown are pure, linear competitive inhibitors: double-reciprocal ($1/v$ vs. $1/S$) plots at different inhibitor concentrations in-

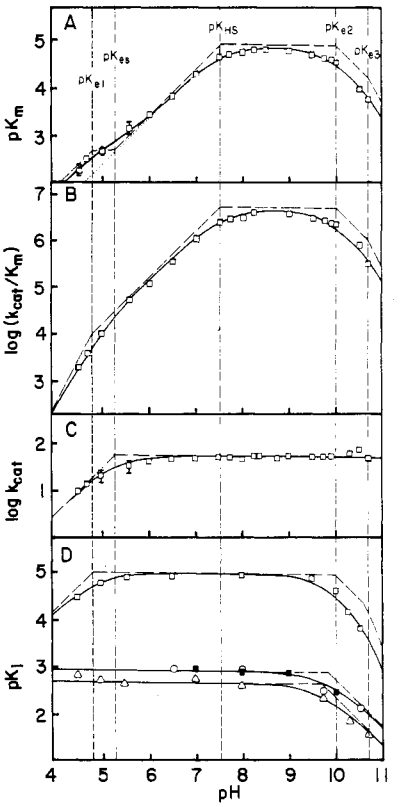

FIGURE 3: pH dependence of the kinetic parameters for the AAP-catalyzed hydrolysis of LPNA and for the inhibition of this hydrolysis by substrate-analogue and transition-state-analogue inhibitors. (A) pH dependence of K_m: theoretical curve (solid line) calculated by using eq 3, with $pK_{e1} = 4.9$, $pK_{e2} = 5.3$, $pK_{e3} = 10.0$, $pK_{e4} = 10.7$, $pK_{HS} = 7.55$, and $K_s = 1.14 \times 10^{-5}$ M. Alternate curve (dotted line) calculated as for theoretical curve except that $pK_{es} = pK_{e1}$. (B) pH dependence of log (k_{cat}/K_m): theoretical curve (solid line) calculated by using eq 5, with the same values used for the constants as in the theoretical curve in (A). (C) pH dependence of k_{cat}: theoretical curve calculated by using eq 4, with $pK_{es} = 5.3$ and $k_2 = 64.5$ s^{-1}. (D) pH dependence of K_i for inhibitors: (□) BuBA; (△) *n*-valeramide; (○) L-leucine; (■) isoamyl alcohol. The theoretical curve for BuBA was calculated by using eq 11, with $pK_{e1} = 4.8$, $pK_{ei} = 5.3$, $pK_{e2} = 10.0$, $pK_{e3} = 10.8$, $pK_{I^0} = 10.6$, $K_{diss} = 2.07$ mM, and $K^* = 225$. The curves for *n*-valeramide, L-leucine, and isoamyl alcohol reflect single ionizations with pK_a equal to either 9.7 (*n*-valeramide) or 9.85 (isoamyl alcohol and L-leucine). Error bars represent ±SE. Where error bars are not shown, the dimensions of the error bars approximate or are smaller than the symbols used.

tersect on the $1/v$ axis, plots of S/v vs. i at different substrate concentrations yield parallel lines (Cornish-Bowden, 1974), and replots of the slopes of $1/v$ vs. $1/S$ plots vs. inhibitor concentration are linear to at least 5 K_i. All of these inhibitors have either *n*-butyl or isopentyl hydrophobic side chains, and all show a decrease in affinity at alkaline pH. The K_i values for *n*-valeramide, isoamyl alcohol, and L-leucine are virtually identical over the range studied, and the pH dependences of all three can be explained by the postulation of one ionizing group ($pK_a \simeq 10$) that must be undissociated in order for inhibitor to be bound. The behavior of the fourth inhibitor shown is strikingly different, however; not only are the K_i values for inhibition by BuBA some 200 times smaller than the values for the other inhibitors but also the slope of the plot of pK_i for BuBA (−3.0) is much steeper in the alkaline range that that for the other inhibitors. The theoretical curve for BuBA was fitted to the data by assuming that three independently ionizing groups with $pK_a = 10.0$, 10.62, and 10.8, respectively, were required to be undissociated in order for inhibitor to be bound. In addition, the plot of K_i for BuBA shows an inflection at pH 4.8 that is not shown by *n*-valer-

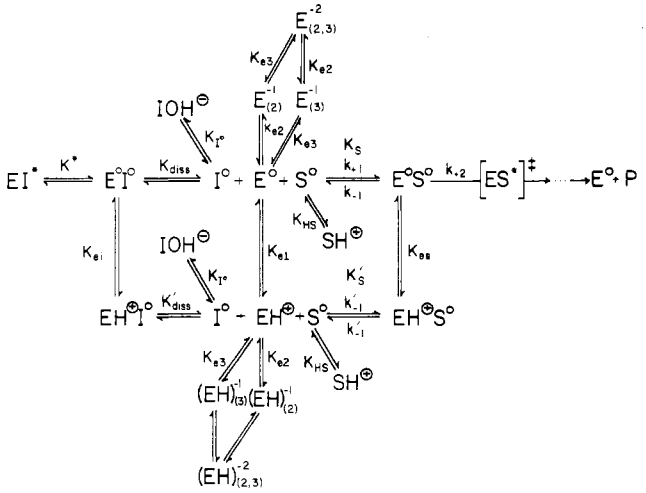

FIGURE 4: Scheme showing proposed analogies between hydrolysis of LPNA by *Aeromonas* aminopeptidase and inhibition of the hydrolysis by BuBA. Lower case subscripts designate specific ionizing groups in either the free enzyme (e1, e2, and e3) or in the enzyme–substrate or enzyme–inhibitor complexes (es or ei), and the constants K_{e1}, K_{e2}, K_{e3}, and K_{es} are the acid dissociation constants for these specific groups (not molecular ionization constants). The subscripts e1, es, and ei refer to the same functional group, ionizing in the free enzyme, in the ES complex, or in the enzyme–inhibitor complex, respectively. K_{HS} is the acid dissociation constant for the substrate α-ammonium group; K_{HI} is the constant for ionization of BuBA by addition of a hydroxyl ion to the boron atom. $K^* = [EI^*]/[E^0I^0]$.

amide, an inhibitor with an identical hydrophobic side chain but a different polar group.

Discussion

It is possible to construct more than one model to explain the pH dependences of K_m, k_{cat}, and K_i for the enzyme–substrate and enzyme–inhibitor systems studied here. The model presented schematically in Figure 4 was chosen for reasons of simplicity; it accounts for the observed pH dependences by using a minimal number of postulated enzyme ionizing groups and assumptions concerning those groups.

In addition to the assumptions explicitly represented in Figure 4 itself, the following two assumptions are required to define the model presented here: (1) The species involved in each reaction step represented by paired arrows are assumed to be effectively at equilibrium ratios; in particular, k_{-1} is assumed to be much larger than k_2, so that $(k_{-1} + k_2)/k_1 \simeq k_{-1}/k_1 = K_s$. (2) Each functional group on the enzyme is assumed to ionize independently of all others.

The rate equation given as eq 1 was obtained from the

$$v = [k_2[E]_t[S]_t/(1 + [H^+]/K_{es})]/\left[[S]_t + K_s\left(\frac{1 + [H^+]/K_{e1}}{1 + [H^+]/K_{es}}\right)(1 + [H^+]/K_{HS})(1 + K_{e2}/[H^+])(1 + K_{e3}/[H^+]) \right] \quad (1)$$

scheme of Figure 4 by using the familiar equilibrium derivation approach but with all inhibitor and enzyme–inhibitor species omitted from the derivation. Equation 1 therefore represents the initial velocity expected in the absence of inhibitor or added product. In this and all following equations, the term $[E]_t$ represents the total enzyme concentration, i.e., the sum of the concentrations of all enzyme-containing species; $[S]_t$ represents the sum of the concentrations of all ionic forms of the substrate.

Provided k_{cat} and K_m are defined as in eq 2 and 3, respectively, eq 1 has the form of the standard Henri–Michaelis–Menten rate equation (eq 4).

$$k_{cat} = k_2/(1 + [H^+]/K_{es}) \quad (2)$$

$$K_m = K_s\left(\frac{1 + [H^+]/K_{e1}}{1 + [H^+]/K_{es}}\right)(1 + [H^+]/K_{HS}) \times (1 + K_{e2}/[H^+])(1 + K_{e3}/[H^+]) \quad (3)$$

$$v = \frac{k_{cat}[E]_t[S]_t}{[S]_t + K_m} = \frac{V_{max}[S]_t}{[S]_t + K_m} \quad (4)$$

Effect of pH on k_{cat}. In the most general terms, there are three possible reasons for the observed decrease in catalytic rate at low pH: (1) The inflection may represent the true pK_a of an enzyme group that ionizes in the enzyme–substrate complex and is required to be in free-base form in order for the rate-limiting step of catalysis to occur. (2) The inflection may represent a change in identity of the rate-limiting step; e.g., with decreasing pH, the protonation of a group required in free-base form for some step, other than the one that is rate limiting at high pH, may have slowed that particular step sufficiently to produce a new rate-limiting step. In this case, the true pK_a of the ionizing group involved will be greater than the "apparent" pK_a (Cleland, 1970). (3) The decrease in rate may reflect nonproductive binding of an ionic form of substrate other than the one hydrolyzed by the enzyme. In a steady-state investigation, however, this third case is kinetically indistinguishable from that of an inactivating protonation occurring only in the ES complex. In view of the boronic acid inhibition data to be discussed below, we have adopted the working hypothesis that the pH 5.3 inflection in the log k_{cat} curve represents the ionization of an enzyme nucleophile (es) required to be in free-base form for attack on the substrate carbonyl to take place. This nucleophilic attack is proposed to be cleanly rate limiting, so that the observed inflection in the log k_{cat} curve corresponds to the true pK_a of the catalytically essential group.

Effect of pH on K_m. The increase in K_m at alkaline pH is interpreted as reflecting a requirement that each of two enzyme groups ionizing between pH 10 and pH 11 be in acidic, undissociated form in order for substrate to be bound. The interactions logically expected for binding and hydrolysis of specific substrates provide at least four categories of interactions that might be involved in the alkaline-limb pH effects. One possibility, in view of the demonstrated major importance of hydrophobic interactions between enzyme and the bulky, hydrophobic N-terminal side chains of specific substrates and inhibitors (Wagner et al., 1972; Baker et al., 1983), is that the undissociated form of at least one of the alkaline-limb ionizing groups may be involved in maintaining the structure of a hydrophobic binding pocket in the enzyme. Support for this idea is provided by the fact that *n*-valeramide, isoamyl alcohol, and L-leucine, three inhibitors different with respect to polar groups, but similar in possessing *n*-butyl or isopentyl hydrophobic side chains, all have K_i values between 1 and 2 mM at pH 8.0 (Figure 3D). In addition to their similar K_i values in the pH region of optimal binding, all three show similar decreases in binding as the pH is increased between 9 and 10.5.

The pH dependence of K_i for these three inhibitors differs from that of K_m, however, in the slopes of the plots at high pH. The binding of substrate (LPNA) appears to require the undissociated form of two enzyme groups, inasmuch as the

slope of a plot of pK_m vs. pH above 10.7 is approximately −2.0, whereas the binding of *n*-valeramide, isoamyl alcohol, or L-leucine is affected by only one ionization, as reflected by a pK_i vs. pH slope of −1 at high pH. This suggests that at least one of the alkaline-limb ionizations affects enzyme–substrate interactions other than those involving the hydrophobic side chain of substrate. The stabilization of the transition states for hydrolysis of substrates by an aminopeptidase logically requires a number of polar interactions between enzyme and the atoms of the scissile bond of substrate (Baker et al., 1983); these interactions are probably preformed to a significant extent in the Michaelis complex and are therefore possible sites for disruption of binding, as are the interactions between the enzyme and the free N-terminal amino group. The decrease in substrate binding at high pH may result, in part, from disruption of interactions between enzyme and the *p*-nitrophenyl ring of the substrate leaving group. This moiety apparently contributes substantially to the affinity between LPNA and the enzyme, inasmuch as LPNA is bound some 260 times more tightly at pH 8.0 than is another readily cleaved substrate, L-leucinamide (Wagner et al., 1972; Prescott & Wilkes, 1976). The interaction of the *p*-nitrophenyl ring with the enzyme, perhaps at the site that binds the side chains of the penultimate residues of substrates studied by Wagner et al. (1972), is therefore another point at which one of the alkaline-limb ionizations may interfere with substrate binding.

In addition to the sharp inflection shown in the pK_m curve above pH 10, there is an increase in K_m as the pH is decreased below pH 8.5. Fitting of the theoretical curve to the data in this region requires that a kinetically significant ionizing group in free enzyme or free substrate have a pK_a value near 7.6. Because this value is not greatly different from the pK_a value of 7.74 for the substrate LPNA, and since a variety of substrate-analogue inhibitors lacking amino groups that ionize in this region do not show similar inflections (Figure 3D), this ionization is assigned to the N-terminal α-amino group of the substrate and is designated by the dissociation constant K_{HS}. This assignment implies that only the unprotonated form of the substrate is bound to the enzyme and that the decrease in concentration of "available substrate" with decreasing pH is the dominant factor in the pH dependence of K_m below pH 8.5.

At pH values of 6.5 and below, the effect of the ionization at pH 7.6 is overlaid by the effects of two additional ionizations, with apparent pK_a values 5.3 and 4.8, that produce a small but reproducible wave in the plot of pK_m vs. pH. The ionization at pH 5.3 is assigned to the group es, the enzyme nucleophile that ionizes in the enzyme–substrate complex and is required to be in free-base form for hydrolysis of bound substrate; the ionization of this group also produces the inflection in the log k_{cat} curve (see above). The concave-downward inflection at pH 4.8 is then assumed to reflect the ionization of this same group in the free enzyme (pK_{e1} = 4.8; pK_{es} = 5.3). Binding of substrate is therefore cooperative with protonation of e1/es, and $K_s'/K_s = K_{es}/K_{e1}$.

The preceding conclusions concerning the significance of the pH dependence of K_m and k_{cat} are embodied in the scheme of Figure 4 and in eqs 1–3, which were derived from the scheme of Figure 4, with enzyme–inhibitor species omitted. The solid curve in the pK_m plot was generated by using eq 3, with the values of ionization constants as indicated above and with K_s assigned the value 1.14×10^{-5} M. In order to illustrate the effect of the cooperativity between substrate binding and protonation of es, an alternate curve (dotted line) has been generated in Figure 3A by using eq 3 with all constants assigned as before, except that pK_{e1} is set equal to pK_{es} (i.e., binding of substrate is assumed to have *no* effect on protonation of e1/es, and vice versa).

Further support for the assignments of the inflections at pH 4.8 and pH 5.3 in the plot of pK_m is provided by the plot of log k_{cat}/K_m in Figure 3B. It has been proposed (Peller & Alberty, 1959; Cleland, 1970) that inflections in plots of log k_{cat}/K_m reflect only ionizations occurring in free enzyme or free substrate, the effects of ionizations occurring in the ES complex having been cancelled algebraically in the ratio. This principle must be applied with some caution, since it does not by any means apply to all of the reasonable reaction schemes that might be constructed for enzyme reactions (Plaut & Knowles, 1972); it does, however, apply to many one-substrate, "rapid-equilibrium" models and can readily be shown to apply to the scheme of Figure 4, since division of eq 2 (k_{cat}) by eq 3 (K_m) yields eq 5, in which k_{cat}/K_m is defined only in terms

$$\frac{k_{cat}}{K_m} = k_2/[K_s(1 + [H^+]/K_{e1})(1 + [H^+]/K_{HS}) \times (1 + K_{e2}/[H^+])(1 + K_{e3}/[H^+])] \quad (5)$$

of free-enzyme and free-substrate ionizations.

The theoretical curve (solid line) in Figure 3B was calculated by using eq 5 and values of the ionization constants as shown in Figure 4. The presence in the log k_{cat}/K_m plot of the inflection at pH 4.8 assigned to pK_{e1} and the absence of the pH 5.3 inflection assigned to pK_{es} are entirely consistent with the assignments as made.

Inhibition by a Transition-State-Analogue Inhibitor. As shown in Figure 3D, BuBA is a far more potent competitive inhibitor than are other small molecules having similar aliphatic groups. The magnitude of the difference is such as to suggest that the boronic acid is bound in a process fundamentally different from the binding of the other molecules studied as possible substrate-analogue inhibitors of *Aeromonas* aminopeptidase. Boronic acids have proved extremely useful in mechanistic studies on the serine proteases. There is a large body of evidence, both from kinetic (Koehler & Lienhard, 1971; Nakatani et al., 1975) and X-ray crystallographic (Matthews et al., 1975) studies, that certain boronic acids containing bulky aliphatic and aromatic groups are bound to α-chymotrypsin and subtilisin BPN′ (Novo) by a process closely analogous to the formation of a hydrolytic transition state from enzyme and substrate. Apparently the first step in the binding is the noncovalent association of enzyme and inhibitor to form a complex resembling the enzyme–substrate Michaelis complex. In the second binding step, the nucleophilic active site serine oxygen adds to the boron of the planar trigonal (Bell et al., 1967; Ross & Edwards, 1967) boronic acid group, to form a tetrahedral adduct resembling the transition state for acylation of enzyme by substrate. In addition, Koehler & Hess (1974) have shown that a borinic acid (general formula R_2BOH) analogue of acetylcholine is a powerful inhibitor of acetylcholinesterase, and probably forms a transition-state-analogue complex with this enzyme as well. Garner (1980) found that various boronic acids are potent inhibitors of porcine pancreatic lipase, apparently binding directly to, or very near, the essential serine residue.

The enzymes reported to date as forming transition-state-analogue complexes with boronic or borinic acids have, without exception, been serine hydrolases. *Aeromonas* aminopeptidase, however, is a zinc metallopeptidase/esterase (Prescott & Wilkes, 1966, 1976; Prescott et al., 1971) that has been shown *not* to contain an essential serine residue (Prescott & Wilkes, 1966). The apparent formation of a transition-state-analogue

complex between *Aeromonas* aminopeptidase and BuBA therefore provides evidence that this type of inhibition by boronic acids may be more general with regard to acyl-hydrolases than previously supposed.

Our conclusion that *Aeromonas* aminopeptidase forms a transition-state-analogue complex with BuBA depends primarily upon two lines of evidence: the differences between the affinity of the enzyme for BuBA and for structurally analogous substrates and substrate-analogue "ground-state" inhibitors, and the similarity between the pH dependence of K_i for BuBA and that of k_{cat} for hydrolysis of LPNA.

At pH 8.0, BuBA ($K_i = 9.6 \times 10^{-6}$ M) is bound to *Aeromonas* aminopeptidase 225 times as tightly as is the substrate-analogue inhibitor, *n*-valeramide ($K_i = 2.16 \times 10^{-3}$ M), and more than 70 times as tightly as the substrate L-leucinamide, which, when used as an inhibitor of the hydrolysis of LPNA at pH 8.0, has a K_i value of 0.7 mM (Baker et al., 1983). The binding constant for BuBA at pH 8.0 is of the same order of magnitude as the K_m observed at this pH for LPNA, the substrate used in this study. The binding of LPNA, however, almost certainly has a large contribution from interactions between the enzyme and the *p*-nitrophenyl leaving-group ring, a structure that has no counterpart in BuBA, *n*-valeramide, or L-leucinamide. The significant comparisons, therefore, are between the substrates and inhibitors that are closely similar in structure, and the most significant comparison is that between BuBA and *n*-valeramide. These two inhibitors have identical aliphatic hydrocarbon side chains, as well as polar groups (dihydroxyboron and carboxamido) that are similar in size and in geometric arrangement of active hydrogens and unshared electron pairs (Koehler & Lienhard, 1971). In view of the structural similarity between *n*-valeramide and the acidic, un-ionized form of BuBA—the predominant form at pH 8.0 (>99%)—it would be difficult to rationalize a difference in K_i of more than 2 orders of magnitude solely on the basis of differences in hydrophobic and hydrogen bonding. It is evident, therefore, that the two inhibitors are bound by fundamentally different processes, with a reasonable hypothesis being that *n*-valeramide is bound in a simple (noncovalent) one-step process (eq 6) and BuBA is

$$E^0 + I^0 \rightleftharpoons E^0I^0 \quad (6)$$

$$\begin{array}{ccccc} E^0 + I^0 & \overset{K_{diss}}{\rightleftharpoons} & E^0I^0 & \overset{K^*}{\rightleftharpoons} & EI^* \\ {\scriptstyle K_{ei}}\downarrow\uparrow & & \downarrow\uparrow{\scriptstyle K_{ei'}} & & \\ EH^+ + I^0 & \rightleftharpoons & EH^+I^0 & & \end{array} \quad (7)$$

$$K_{diss} = \frac{[E^0][I^0]}{[E^0I^0]} \quad (8)$$

$$K^* = \frac{[EI^*]}{[E^0I^0]} \quad (9)$$

bound in a two-step process (eq 7) analogous to that proposed for binding of boronic acids to serine proteases (Nakatani et al., 1975). In this two-step process (eq 7), the second, unimolecular binding step is proposed to involve addition of an enzyme nucleophile to an electron-deficient orbital of the inhibitor boron atom.

If the nucleophile involved in the attack on the boronic acid is the same nucleophile that attacks the substrate carbonyl carbon, and if the decrease in k_{cat} at low pH reflects the protonation of this nucleophile (see above), then the inhibitory capability of BuBA should show a decrease at low pH, analogous to the decrease in k_{cat} in this region. This is, in fact, reflected in Figure 3D. The value of pK_i decreases with decreasing pH below pH 6.0, apparently as a result of protonation of an ionizing group with pK_a near 4.8.

In addition to the pH effects observed in the acid range for inhibition by BuBA, there is a sharp decrease in inhibitory effectiveness above pH 10 (Figure 3D). At low pH, the plot of pK_i for BuBA vs. pH resembles the corresponding plot of log k_{cat}; at high pH, however, the plot of pK_i bears a strong resemblance to the plot of pK_m vs. pH in Figure 3A. The pK_i and pK_m curves show similar sharp decreases in affinity in the same alkaline pH region, although the slope of the pK_i curve above the inflection (= −3.0) indicates that K_i is affected by ionization of three groups with pK_a in this region, rather than by two, as in the case of K_m. This is to be expected if the first step in binding of the boronic acid requires the undissociated form of the same two enzyme groups (e2 and e3) that are required to be undissociated for the binding of substrate, and if the ionized form of BuBA ($pK_a = 10.62$) is noninhibitory. In terms of the two-step binding scheme presented for BuBA (eq 7 and Figure 4), the alkaline-limb pH dependence of pK_i for BuBA may be seen as reflecting predominantly the pH effects on the first (substrate-analogue) binding step and the acidic-limb pH dependence as reflecting the effect of an enzyme ionization upon the second (transition-state-analogue) binding step.

Proposed analogies between inhibition of *Aeromonas* aminopeptidase by BuBA and the binding and hydrolysis of substrate are represented schematically in Figure 4. Equation 10, derived from the scheme of Figure 4 (with all inhibitor-containing species included) describes the expected initial rates in the presence of an inhibitor binding in a two-step process, with a second step requiring the free-base form of a group (ei) ionizing in the EI complex.

$$v_{inhib} = k_{cat}[E]_t[S]_t \Big/ \left[[S]_t + K_m \left[1 + \frac{[I]_t}{K_{diss}} \left(\frac{1}{1 + K_{I^0}/[H^+]} \right) \left(\frac{1 + [H^+]/K_{ei} + K^*}{1 + [H^+]/K_{el}} \right) \times \left(\frac{1}{1 + K_{e2}/[H^+]} \right) \left(\frac{1}{1 + K_{e3}/[H^+]} \right) \right] \right] \quad (10)$$

In eq 10, K_m and k_{cat} are defined by eq 2 and 3, respectively, and have been substituted for the equivalent expressions. Provided K_i is defined as in eq 11, eq 10 will have the form of eq 12:

$$K_i = K_{diss} \left(\frac{1 + [H^+]/K_{el}}{1 + [H^+]/K_{ei} + K^*} \right) (1 + K_{e2}/[H^+]) \times (1 + K_{e3}/[H^+])(1 + K_{I^0}/[H^+]) \quad (11)$$

$$v = \frac{V_{max}[S]_t}{[S]_t + K_m(1 + [I]_t/K_i)} \quad (12)$$

In eq 11, K_{diss} represents the K_i value that would be observed if all ionizing groups were completely and simultaneously in the form required for the first step, $E^0 + I^0 \rightleftharpoons E^0I^0$, to take place and if the second step, $E^0I^0 \rightleftharpoons EI^*$, did not take place. In calculating the theoretical values of K_i for BuBA, it was assumed that if the second step ($E^0I^0 \rightleftharpoons EI^*$) did not take place, BuBA would bind to the enzyme in the same way and with essentially the same affinity as does *n*-valeramide. The value of K_i for *n*-valeramide at pH 8.0 and below was therefore taken as the value of K_{diss} in eq 7. When the values of K_i for

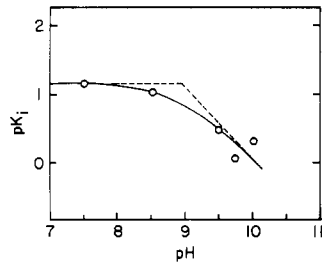

FIGURE 5: Plot of pK_i vs. pH for the inhibition by boric acid/KOH buffer of the *Aeromonas* aminopeptidase catalyzed hydrolysis of LPNA. Values of pK_i were calculated by using the total boron concentration (boric acid plus borate anion).

BuBA and *n*-valeramide at pH 8.0 are substituted for K_i and K_{diss}, respectively, in eq 11, (with pK_{e1} assumed equal to 4.8 and pK_{ei} assumed equal to 5.3), the value obtained for K^* is 225, indicating that the enzyme–inhibitor complex having the boron atom tetrahedral is some 225 times more stable than the substrate-analogue complex with boron in planar trigonal configuration.

Experimental support for the idea that ionization of the boronic acid abolishes the tight binding of BuBA to the enzyme is found both in the literature and in the present results. Philipp & Bender (1971) found that the inhibition of subtilisin by various ring-substituted phenylboronic acids decreased at alkaline pH and that, in general, the apparent pK_a controlling this decrease approximates the pK_a of the particular boronic acid. In the present study, the inhibition of LPNA hydrolysis by boric acid was also found to decrease at high pH, with the decrease following the ionization curve of boric acid (Figure 5). Boric acid and the dihydroxyboron group of BuBA bind to *Aeromonas* aminopeptidase either at the same locus or at substantially overlapping loci (Baker et al., 1983), and it seems reasonable to conclude that they have similar binding mechanisms. Ionization of BuBA would then be expected to have an effect on K_i similar to that seen with boric acid, but at a higher pH corresponding to the pK_a of BuBA.

The scheme of Figure 4 indicates that the ionized form of BuBA forms neither an EI nor an EI* complex with the aminopeptidase. It is not clear from the present data whether ionization of the inhibitor actually prevents noncovalent (EI) binding to the enzyme, but it seems almost certain that a tetrahedral boronate anion, even if bound noncovalently to the enzyme as in the first step proposed here, would not undergo the second step (nucleophilic addition) to form the most stable complex (Koehler & Hess, 1974; Hess et al., 1975). Given the value estimated above for K^*, and with the assumption that binding of the boronate anion is approximately as tight as the first-step (noncovalent) binding of the neutral acid, then prevention of the second step in binding would be expected to increase K_i by a factor of approximately 226, which is tantamount to abolition of the inhibition. A one-step inhibition by the boronate anion to equal the contribution from two-step binding of that fraction of the inhibitor still present as the nonionized, planar trigonal acid would require pH $\simeq$13; thus, the question of whether the boronate anion is bound or not should have no significant bearing on the interpretation of the results between pH 4.5 and 10.7. In either case, ionization of the boronic acid should effectively abolish inhibition in this pH range. An apparently analogous situation has been found by Andersson et al. (1982) in a study of an enzyme–inhibitor system that involves a different aminopeptidase and a different chemical class of inhibitor but, like the systems discussed above, appears to involve formation of a covalent adduct between inhibitor and an enzyme nucleophile. Andersson et al. concluded that the free aldehyde form of L-leucinal is the form tightly bound to microsomal aminopeptidase from porcine kidney and that the inhibitory effectiveness of the amino acid aldehyde is decreased to the extent that the planar trigonal aldehyde group is converted to the tetrahedral hydrate.

One of the characteristics of an ideal transition-state-analogue inhibitor is that the pH dependence of inhibition by such molecules is expected to resemble the pH dependence of the catalytic rate constant for normal substrates (Palmer et al., 1982). The expected similarity between the two pH dependences does not, however, necessarily imply that for all systems the two curves will be identical to the extent of having their inflections at exactly the same pH. The model presented in Figure 4, in fact, supplies an example of a system in which the inflection in the log k_{cat} curve would not be expected to be replicated exactly in the pK_i curve for the transition-state-analogue inhibitor, even though the acid-range inflections in both curves are due to ionization of the same group. In Figure 4, the covalent enzyme–inhibitor complex EI* is shown as being analogous to the transition state for the rate-limiting step of substrate hydrolysis; E^0I^0 and EH^+I^0 are shown as analogous to E^0S^0 and EH^+S^0, respectively. The analogies between the overall patterns of the proposed reaction schemes, and between the chemical interactions proposed as taking place in the individual steps being compared, are quite close. The pH dependences predicted by the scheme for log k_{cat} and pK_i are significantly different, however. Whereas the pH dependence of log k_{cat} will reflect the ionization of an enzyme nucleophile (es) ionizing in the enzyme–substrate complex with $pK_a = pK_{es}$ (i.e., it reflects the pH dependence of the relative steady-state concentrations of E^0S^0 and EH^+S^0), the value of pK_i in the acid range will be controlled by the ionization of this same group (now denoted e1) *in the free enzyme*, and the significant ratio is that between the steady-state concentrations of E^0 and EH^+. (Perfect analogy, based on the scheme of Figure 4, would lead one to expect that pH-induced changes in pK_i would reflect changes in the relative steady-state concentrations of E^0I^0 and EH^+I^0, the species analogous to E^0S^0 and EH^+S^0, respectively.) The reason for this breakdown of analogy between the two pH dependences can be traced to one important difference between transition-state complexes and their analogues: Whereas the steady-state concentrations of transition states are extremely small with respect to total enzyme concentration, and can therefore be neglected in the writing of distribution equations, the enzyme–inhibitor transition-state-analogue complexes accumulate to a considerable extent at steady state and are thus not negligible. This characteristic of the analogues—the feature that renders them highly useful by making them amenable to direct study—also therefore limits the extent to which the kinetics of inhibition by transition-state-analogue inhibitors can be analogous to the kinetics of catalysis of substrate reactions.

This principle is illustrated mathematically in eq 11, in which the terms affecting the acidic-range pH dependence of K_i are collected in the first parentheses. The ionization of e1 is seen to be capable of affecting K_i as soon as the value of $[H^+]/K_{e1}$ becomes significant with respect to 1, but the presence of K^* in the denominator will tend to damp out the effect of ionization of ei until the pH is sufficiently low that $[H^+]/K_{ei}$ becomes significant, not with respect to 1, but with respect to $1 + K^*$ ($\simeq$226). The presence of K^* in the denominator is due to the fact that the steady-state concentration of EI* is nonnegligible with respect to total enzyme; compare this denominator with that of the first parenthetical term in eq 3, in the derivation of which equation the steady-state

concentration of the transition state analogous to EI* was assumed to be negligible.

Consider the portion of Figure 4 that deals with inhibitor binding: at values of $[I^0]$ in the range of the overall K_i determined from initial rate studies, almost all of the enzyme will be present either as EI* or as one of the "free" enzyme species E^0 or EH^+; i.e., the first binding equilibrium will be far to the right toward free enzyme and free inhibitor. Thus, a large value for K^* will ensure that the relative distribution between E^0I^0 and EH^+I^0 will carry little weight in determining the value of K_i, except at pH values sufficiently low that the ratio $[EH^+I^0]/[E^0I^0]$ approximates K^*; in that case, the relative concentration of EH^+I^0 will carry a weight comparable to that of EI*. This principle should be applicable to all enzyme–inhibitor systems in which tight binding of inhibitor depends upon a relatively weak initial binding equilibrium, followed by a second, unimolecular step that produces a much more stable complex, this second step being dependent upon a particular ionization state of an functional group. Such systems have been described by Nakatani et al. (1975) and Hess et al. (1975) for boronic acids and serine proteases and by Frieden et al. (1980) and Kurz & Frieden (1983) for adenosine deaminase. For all such systems, any inflection in the pK_i vs. pH curve, due to ionization of the enzyme group that is essential for the second step, will occur at a pH value approximately equal to the pK_a of that group in the free enzyme. Only in the special case in which the ionization of the catalytically essential group is unaffected by binding of substrate or inhibitor ($pK_{ei} = pK_{es} = pK_{el}$), will the position of the inflection be numerically equal to pK_{es}.

The results shown in Figure 3D for inhibition by BuBA are entirely consistent with the principle set forth above. In the acidic range, the plot of pK_i for BuBA shows an apparent pK_a of 4.8, identical with the value obtained for pK_{el} from fitting of eq 3 to the pK_m data, rather than having an apparent pK_a at pH 5.3, the value obtained for pK_{es} from both k_{cat} and K_m data. The data in Figure 3D therefore yield the value of pK_{el} for the free enzyme but provide no direct information concerning the value of pK_{ei} for the (noncovalent) complex between BuBA and the enzyme.

Possible Identities of Enzyme-Ionizing Groups. Much of the preceding points to the existence in *Aeromonas* aminopeptidase of a catalytically essential nucleophile that ionizes near pH 5.3. The chemical modification studies of Mäkinen et al. (1982b) have shown that whereas a carboxylic acid residue is essential to the activity of the enzyme, two histidine residues can be modified without a decrease in activity. Evidently the catalytically essential group with pK_a near 5.3 is either an aspartyl or glutamyl carboxyl. The inactivation of the aminopeptidase by iodine and by acetic anhydride (Baker & Prescott, 1980) and by nitration and diazotization (Mäkinen et al., 1982b), together with the partial reactivation of the acylated enzyme by neutral hydroxylamine (Baker & Prescott, 1980), implicates tyrosine as an essential residue. It remains to be determined, by further chemical modification studies and spectral studies of possible pH-dependent conformational changes, whether one or more of the kinetically significant ionizing groups with pK_a between 10 and 11 are tyrosine residues.

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Registry No. LPNA, 4178-93-2; BuBA, 4426-47-5; *n*-valeramide, 626-97-1; Leu, 61-90-5; isoamyl alcohol, 123-51-3; aminopeptidase, 9031-94-1.

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Protocatechuate 3,4-Dioxygenase: Comparative Study of Inhibition and Active-Site Interactions of Pyridine *N*-Oxides†

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ABSTRACT: The binding of 2-hydroxypyridine *N*-oxide (HYPNO) and a series of structural analogues to protocatechuate 3,4-dioxygenase (PCD) has been investigated by using kinetic and spectral techniques. HYPNO binds less tightly to PCD than 2-hydroxyisonicotinic acid *N*-oxide (HINANO), a compound designed to mimic structural features of species along the PCD reaction pathway, which we have previously shown to bind to the enzyme in a kinetically irreversible manner [May, S. W., Oldham, C. D., Mueller, P. W., Padgette, S. R., & Sowell, A. L. (1982) *J. Biol. Chem. 257*, 12746–12751]. HYPNO was found to be a time-dependent inhibitor of PCD. The rate constants for both binding (100 M^{-1} s^{-1}) and dissociation (3×10^{-4} s^{-1}) of HYPNO were found to be 4 orders of magnitude less than those for 3-fluoro-4-hydroxybenzoic acid (3-FHB), the best freely reversible competitive inhibitor of PCD. Although inhibition by HYPNO is not freely reversible, it can be slowly reversed by a simple displacement with 3-FHB. This is in contrast to the inhibition by HINANO, whose binding to PCD is reversed only by denaturing the enzyme. The displacement of HYPNO by 3-FHB was examined spectrophotometrically and found to consist of rapid formation of a species that is spectrally distinct from the complex of PCD with either inhibitor alone, followed by a slow change to give the spectrum of the PCD·3-FHB complex. Analysis of the inactivation and spectral data, along with determination of the concentrations of both inhibitors immediately after the initial rapid change, is consistent with formation of a ternary PCD·HYPNO·3-FHB complex. The initial binding and time dependence of inhibition of a series of related ring-substituted pyridine *N*-oxides were also studied. The presence of a ketonizable group adjacent to the *N*-oxide causes slow, tight binding of the type seen with HYPNO and HINANO. The spectral characteristics of the complexes of HYPNO and HINANO with PCD differ markedly from those of the halohydroxybenzoate–PCD complexes and this may reflect differences in the ligation environment of the active-site iron in these species.

Among the reactions catalyzed by dioxygenases is the oxygenolytic cleavage of aromatic rings, and the mechanism of this process is a subject of much current interest. One of these enzymes, protocatechuate 3,4-dioxygenase (PCD),[1] which catalyzes the intradiol cleavage of protocatechuic acid, has been isolated from a number of microbial genera (Stanier & Ingraham, 1954; Wells, 1972; Hou et al., 1976; Durham et al., 1980; Bull & Ballou, 1981), with the crystalline enzyme from *Pseudomonas aeruginosa* being the most extensively studied (Fujisawa & Hayaishi, 1968). One method of investigating the enzymatic mechanism is by studying the interaction of various types of inhibitors with the enzyme active site. We, and others, have studied the 3-halo-4-hydroxybenzoates and have found them to be potent rapidly reversible PCD inhibitors (May et al., 1978; May & Phillips, 1979; Felton et al., 1978; Fujisawa et al., 1971, 1972a,b; Que et al., 1977; Keyes et al., 1978; Tatsuno et al., 1978; Nakata et al., 1978). When complexed with these inhibitors, the enzyme gives a characteristic spectrum with a visible maximum at 420 nm. Kinetic and resonance Raman investigations carried out in this and other laboratories have provided evidence that in the initial binding step, the *p*-OH of substrates or inhibitors interacts directly with the essential iron atom of the enzyme via Fe–O ligation. In the case of substrates, subsequent ketonization of the *m*-OH allows oxygen attack at the adjacent carbon to give an α-ketohydroperoxide, followed by collapse to product (Scheme I).

In order to obtain further support for this mechanism, we have recently studied a compound designed to mimic the ketonized transient species (cf. VI and VIII of Chart I) of the reaction pathway (May et al., 1982). This compound, 2-hydroxyisonicotinic acid *N*-oxide (HINANO, VIIa) was found to be an extremely potent, kinetically irreversible inhibitor and an active-site titrant, although it is not a substrate for PCD. Furthermore, denaturation of the enzyme–HINANO complex released virtually all of the bound inhibitor in an active form, indicating that covalent binding of HINANO to the enzyme is unlikely. Inhibition with similar characteristics has been observed in studies with "transition-state analogues" of various enzymes (Frieden et al., 1980; Wolfenden, 1976). The

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[1] Abbreviations: PCA, protocatechuic acid; PCD, protocatechuate 3,4-dioxygenase [protocatechuate:oxygen 3,4-oxidoreductase (decyclizing) EC 1.13.11.3]; HINANO, 2-hydroxyisonicotinic acid *N*-oxide; 3-FHB, 3-fluoro-4-hydroxybenzoic acid; 3-ClHB, 3-chloro-4-hydroxybenzoic acid; CINANO, 2-chloroisonicotinic acid *N*-oxide; NANO, nicotinic acid *N*-oxide; INANO, isonicotinic acid *N*-oxide; MINANO, 2-mercaptoisonicotinic acid *N*-oxide; 3-XHB, 3-halo-4-hydroxybenzoic acid; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)-aminomethane.

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