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Use of Secondary Isotope Effects and Varying pH To Investigate the Mode of Binding of Inhibitory Amino Aldehydes by Leucine Aminopeptidase†

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ABSTRACT: K_i values for leucine aldehyde, a competitive inhibitor of leucine aminopeptidase, vary with pH in a manner compatible with binding of uncharged inhibitor. The pH dependence of k_{cat}/K_m suggests likewise that the substrate leucine *p*-nitroanilide is productively bound as the uncharged species. Comparison of pK_a values of the model compounds aminoacetone and aminoacetal indicates that the equilibrium constant for hydration of amino aldehydes is reduced by a factor of about 2 when a proton is lost from the α-ammonium group near pH 8. Effects of deuterium substitution at C-1 on equilibrium binding of leucine aldehyde were determined with immobilized enzyme and inhibitors doubly labeled with radioisotopes. The observed isotope effect (K_D/K_H) is approximately unity, suggesting that leucine aldehyde combines with the enzyme as an oxygen adduct, not as the intact aldehyde.

The principle of transition-state analogy has been helpful in designing reversible enzyme inhibitors that much surpass substrate analogues in their binding affinities. In cases where an inhibitor of this kind could be bound in any of several possible forms, the actual mode of binding that is observed can be helpful in attempting to distinguish between possible mechanisms (Wolfenden, 1980). Simple amino aldehydes (Andersson et al., 1982), and also amino acid hydroxamates (Chan et al., 1982; Coletti-Previero et al., 1982; Wilkes & Prescott, 1983), have recently been found to serve as strong inhibitors of leucine aminopeptidases from kidney. Amino aldehydes also prolong the analgesic effects of enkephalins and related peptides in vivo, perhaps by preventing their degradation (Davis et al., 1983). This paper describes experiments designed to secure structural information about the binding of amino aldehydes by cytosolic leucine aminopeptidase.

In aqueous solution, α-amino aldehyde cations exist as minor (ca. 2.5%) constituents of an equilibrium mixture consisting mainly of their covalent hydrates or *gem*-diols (Andersson et al., 1982). Influences of pH on inhibition, and on productive binding of substrates, were examined first. The effect of pH on covalent hydration of amino aldehydes (and the corollary influence of hydration on ionization) was then estimated by determining the pK_a values of a model ketone and acetal.

To distinguish between modes of binding, it seemed possible to exploit the known effect of deuterium substitution in the aldehyde group in promoting equilibrium addition of nucleophiles (Lewis & Wolfenden, 1977a). If an inhibitory aldehyde were taken up by the enzyme intact, then its binding would be expected to reflect, almost in full, the 1.37-fold difference in equilibria of hydration between aldehydes and 1-^{2}H-labeled

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aldehydes (Lewis & Wolfenden, 1977b). Because of the greater difficulty of stripping covalently bound water from the deuterioaldehyde, the protioaldehyde would appear to be bound substantially more tightly at equilibrium. Attempting to test this possibility for leucine aminopeptidase, we first compared the inhibitory effects of leucinal (leucine aldehyde) with those of [1-^{2}H]leucinal. K_i values appeared to be similar for the two inhibitors but were not sufficiently precise to permit conclusions to be drawn with confidence (Andersson et al., 1982). To obtain a more exact comparison of the binding affinities of protio- and deuterioaldehydes, radioisotopic labels were therefore introduced into these compounds to permit simultaneous comparison of their uptake by leucine aminopeptidase immobilized on Sepharose CL-4B.

Materials and Methods

Soluble Leucine Aminopeptidase. To prepare the Zn_6Mn_6 form of the enzyme, cytosolic leucine aminopeptidase (type III-CP, obtained from Sigma as an ammonium sulfate suspension) was first mixed with an equal volume of tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (10^{-2} M, pH 8.0) containing 10^{-4} M $ZnCl_2$, allowed to stand for 2 h at 37 °C, and then dialyzed against the same buffer overnight at 4 °C according to the procedure of van Wart & Lin (1981). The dialyzed enzyme (approximately 1.1 mg/mL) was then mixed with an equal volume of Tris-HCl buffer (10^{-2} M, pH 8.0) containing 10^{-2} M $MnCl_2$ and allowed to stand at 37 °C for 2 h before use in kinetic experiments.

Immobilization of Leucine Aminopeptidase. Sepharose CL-4B was activated by treatment of 5 mL of the gel with CNBr (0.67 g) following a published procedure (March et al., 1974). To 6.6 mg of leucine aminopeptidase (LAP) (dialyzed against 3 × 3.5 L of 0.1 M $NaHCO_3$ overnight at 4 °C) dissolved in 11 mL of 0.1 M $NaHCO_3$ was added 5 mL of CNBr-activated agarose, and the mixture was allowed to react overnight at 4 °C. After coupling, the gel was washed exhaustively with 0.1 M $NaHCO_3$ and with 10 mM Tris-HCl, pH 8, containing 0.1 mM $ZnCl_2$ (50 mL). The normal zinc content of immobilized LAP was restored by treatment of the immobilized enzyme in 10 mM Tris-HCl, pH 8, containing 0.1 mM $ZnCl_2$ for 2–3 h at 37 °C. The enzyme preparation was then washed on a glass filter with 10 mM Tris-HCl, pH 8, and stored in this buffer at 4 °C prior to use. Immobilized LAP was activated by treating the Sepharose-bound enzyme with 10 mM Tris-HCl, pH 8, containing 5 mM $MnCl_2$, at 37 °C for 3–4 h. Activated immobilized LAP was stored in this buffer at 4 °C. Enzyme activity was measured at 25 °C in 10 mM Tris-HCl, pH 8, containing 1 M NaCl, 5 mM $MnCl_2$, 1 mM L-leucine *p*-nitroanilide, and 0.7% dimethyl sulfoxide (Me_2SO) (v/v); the final assay volume was 3 mL. The activity of immobilized LAP was found to increase 26-fold as a result of treatment with $MnCl_2$, when the activity of $ZnCl_2$-treated immobilized enzyme was used as a reference. The activity of soluble LAP has been reported to increase about 10–13-fold by similar treatment (van Wart & Lin, 1981). Reference Sepharose, containing no immobilized enzyme, was treated identically except for the omission of aminopeptidase from the coupling reaction. Preparations of immobilized LAP were also treated with Tris-HCl (0.5 M, pH 8) containing 5 mM $MnCl_2$ for 3 h at room temperature, in order to block any activated groups remaining on the gel after immobilization and treatment of the immobilized enzyme preparation with $ZnCl_2$. The enzyme preparation treated with a high concentration of Tris-HCl was also activated in buffer containing $MnCl_2$ as described above. The resulting immobilized enzyme preparation was found to bind L-leucinal to the same extent as did LAP covalently bound to a gel that had not been treated with Tris-HCl buffer. After the gel was extensively washed on a glass filter, immobilized LAP contained 0.8–1.4 mg of protein bound/mL of settled gel, as estimated by UV measurements at 280 nm [$\epsilon_{280} = 4 \times 10^5$ M^{-1} cm^{-1} (van Wart & Lin, 1981)].

Synthesis of Radioactive L-Leucinal. L-Leucinal was synthesized from L-leucinol by enzymatic oxidation with alcohol dehydrogenase (ADH) by a modified version of the published method (Andersson & Wolfenden, 1982), using polymer-bound hydrazine to trap the aldehydes formed. This obviates the need for the extraction and chromatography steps that had been needed to purify semicarbazones and recover aldehydes from the crude reaction mixture. Bio-Gel P6 (10 g) was first treated with hydrazine (4.7 M) in 30 mL of water for about 8 h at 60 °C. This procedure yields derivatized polyacrylamide containing hydrazido groups attached to the polymer. The degree of substitution of the polymer, determined by the method of Inman & Dintzis (1969), was 0.76 mequiv of hydrazine per gram of wet gel. To a solution (10 mL) containing 75 mM sodium pyrophosphate buffer, pH 8.7, was added a mixture of [1-^{14}C]-L-leucinol and [1-^{2}H,4,5-^{3}H]-L-leucinol, oxidized nicotinamide adenine dinucleotide (NAD^+) (15 mg), flavin mononucleotide (FMN) (200 mg), ADH (44 mg), and 12 g of wet hydrazine–Bio-Gel P6. This mixture was allowed to react for 24 h in the dark at room temperature, and then additional NAD^+ (13 mg) and ADH (53 mg) were added to the reaction mixture. After reaction for another 4 days in the dark at room temperature, the gel was washed exhaustively on a glass filter with 0.1 M NaCl, H_2O, 0.1 M $NaHCO_3$, H_2O, and H_2O/MeOH (1/1) (100 mL). Aldehyde was recovered by treatment of the gel with 0.1 N HCl (10 mL) in 50% aqueous methanol containing formaldehyde (260 mM). After 7.5 h at room temperature, the gel was washed on a glass filter with 0.1 N HCl in 50% aqueous methanol. The filtrate was collected, and the gel was mixed with another 10 mL of 0.1 N HCl in 50% aqueous methanol containing 260 mM formaldehyde. This procedure of eluting polymer-trapped aldehyde stepwise was repeated 4 times. Filtrates containing aldehyde were pooled, and the solution was evaporated to dryness while the temperature of the water bath was kept below 30 °C. The residue was dried over P_2O_5 under vacuum. Radioactive L-leucinal prepared in this manner was free of detectable chemical or radiochemical impurities as judged by thin-layer chromatography on cellulose in 1-butanol/H_2O/acetic acid (50/30/20); $R_f = 0.97$.

Results

Influences of pH on Catalysis and Inhibition. To determine the effect of pH on the catalytic efficiency of Zn_6Mn_6 leucine aminopeptidase, reaction rates were examined as a function of varying concentrations of the substrate leucine *p*-nitroanilide at 25 °C in the presence of Tris-HCl and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–KOH buffers (0.01 M) adjusted to ionic strength 1.0 by addition of KCl. Double-reciprocal plots yielded apparent second-order rate constants that increased with increasing pH to a maximum near pH 9 (Figure 1, upper curve) (see also Lin & van Wart, 1982). For purposes of discussion, the theoretical line in Figure 1 shows the relative abundance in solution of the conjugate base of an acid with $pK_a = 7.93$, indicated by the arrow pointing downward.

Similar studies, carried out in the presence of the competitive inhibitor L-leucinal, gave the results shown in bottom part of Figure 1. Inhibition became more effective with increasing pH. The theoretical line shows the pH dependence expected for an inhibitory process requiring the conjugate base of an

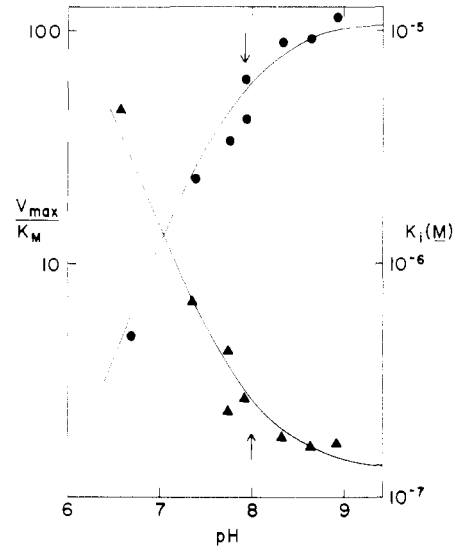
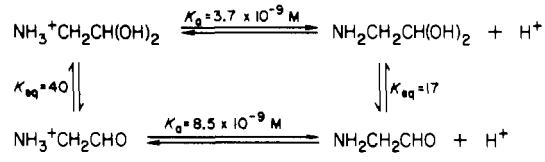


FIGURE 1: Influence of changing pH on the rate of hydrolysis (V_{max}/K_m) of L-leucine *p*-nitroanilide by kidney cytosolic leucine aminopeptidase (circles) and on K_i values observed for competitive inhibition by L-leucinal (triangles), at 25 °C in the presence of Tris-HCl and HEPES–KOH buffers (0.01 M) adjusted to ionic strength 1.0 by addition of KCl.

Scheme I



acid with pK_a = 8.02, as indicated by the arrow pointing upward.

Hydration vs. Ionization of Amino Aldehydes. To obtain information about the influence of ionization on the equilibria of hydration of inhibitory amino aldehydes, it was desirable to compare the pK_a of the conjugate acid of an amino aldehyde with that of its covalent hydrate. These equilibria, linked as shown in Scheme I, were too rapid for their microconstants to be determined directly. The equilibrium constant for hydration of protonated aminoacetaldehyde determined by NMR at 25 °C was 39.6 (Andersson et al., 1982). The corresponding value for the conjugate base could not be measured with certainty in the presence of competing condensation reactions, which were found to occur in alkaline solution at high aldehyde concentrations.

To circumvent these problems, we decided instead to measure acid dissociation constants of stable model ammonium compounds, using an acetal as a model for the amino aldehyde hydrate and a ketone as a model for the intact amino aldehyde. Determined potentiometrically with KOH at 25 °C and ionic strength 0.015, we observed apparent pK_a values of 8.43 ± 0.07 for aminoacetaldehyde dimethyl acetal hydrochloride and 8.07 ± 0.03 for aminoacetone hydrochloride. On the basis of this 2.3-fold effect on K_a, Scheme I suggests that hydration of aminoacetaldehyde should be approximately 2.3-fold less favorable than hydration of its protonated species. The latter was associated with an equilibrium constant of 39.6 (Andersson et al., 1982), so that the equilibrium constant for hydration of uncharged aminoacetaldehyde is presumably about 17 (Scheme I).

Properties of Immobilized Leucine Aminopeptidase. Assayed in Tris-HCl buffer (0.01 M, pH 8.34) containing $MnCl_2$ (5×10^{-3} M), KCl (1 M), L-leucine *p*-nitroanilide (10^{-3} M), and Me_2SO (1% v/v) at 25 °C, immobilized leucine aminopeptidase activated as described under Materials and Methods exhibited approximately 80% of the activity reported for the same enzyme preparation before immobilization. Enzyme activity was measured by following the change in absorbance at 405 nm ($\Delta\epsilon_{405}$ = 9900 M^{-1} cm^{-1}) (Royer & Andrews, 1973). A double-reciprocal plot of the rate measured as a function of varying substrate concentration yielded a K_m value of 2.2 $\times 10^{-4}$ M for the Sepharose-bound enzyme, slightly lower than a value of 10^{-3} M reported for the soluble enzyme under similar conditions (van Wart & Lin, 1981) and confirmed in the present experiments.

Influence of Deuterium on the Binding of Inhibitory Aldehydes by Immobilized Enzyme. Sepharose-bound leucine aminopeptidase (0.7 mL of gel containing enzyme activated by treatment with $MnCl_2$ as described under Materials and Methods) was mixed with 4.8 mL of a solution containing Tris-HCl buffer (10^{-2} M, pH 8.34), KCl (1 M), $MnCl_2$ (5×10^{-3} M), and a mixture of isotopically labeled L-leucinals. The mixture was gently agitated for 1 h at 25 °C and centrifuged at the same temperature, and the contents of ^3H and ^{14}C were then determined in an aliquot (1 mL) of the supernatant fluid. These were compared with the results obtained in an experiment using control gel, prepared identically to the enzyme gel except for the omission of protein (see Materials and Methods). The difference between the two results was used to determine the amount of radioactivity of each kind bound by the protein. This was then compared with the ratio of isotopically labeled aldehydes originally present in solution in order to obtain an indication of the difference between their specific binding affinities.

Inhibitor was introduced as a mixture of [1-^{2}H,4,5-^{3}H]-L-leucinal and [1-^{14}C]-L-leucinal in two sets of experiments. In another set of experiments, the combination of stable and radioactive isotopes was reversed during synthesis, so that tritium was present in the side chain of leucinal and ^{14}C was present in the carbonyl group of 1-deuterioleucinal. In a typical experiment, the binding mixture contained ^3H-labeled deuterioleucinal (7.03×10^{-8} mol; 7.1×10^4 dpm) and ^{14}C-labeled protioleucinal (3.45×10^{-8} mol; 1.6×10^4 dpm), and 0.41 mg of protein was present in the gel. Of the deuterioaldehyde originally present, 3.99×10^3 dpm was adsorbed by the enzyme (1.76×10^4 dpm for the enzyme gel minus 3.06×10^3 dpm for the blank gel). Accordingly, the $^3H/^{14}C$ ratio of leucinal bound by the enzyme in the experiment was 4.3 ± 0.6, as compared with 4.5 ± 0.3 for the aldehyde originally present in solution. The total amount of aldehyde taken up by the enzyme corresponded to approximately 37% of the sites that would be available if the protein were a hexamer of mol wt 320 000 (van Wart & Lin, 1981), with six subunits behaving identically.

To minimize counting errors, samples were subjected to very long periods of analysis by scintillation counting. The entire synthesis was carried out 3 times (once with the isotopes reversed), and the binding experiment was performed on five separate occasions, consistently yielding values for $K_i(D)/K_i(H)$ in the neighborhood of unity, with a mean value of 1.04 ± 0.06.

Discussion

In view of the critical need for an amino group in substrates of leucine aminopeptidase, it is unlikely that ionization of the amino function would leave their binding by the enzyme unaffected. The inflections observed near pH 8, in plots of V_{max}/K_m for the substrate leucine *p*-nitroanilide and K_i for L-leucinal (Figure 1), are in the neighborhood of the pK_a values

of these compounds and presumably reflect a requirement that substrate and ligand be present in solution in unprotonated form for binding to occur. These results do not eliminate the possibility that, during formation of the enzyme–ligand complex, the ligand may have become protonated with accompanying loss of a proton (or uptake of a hydroxide ion) by a group on the protein.

If an inhibitory aldehyde is taken up in its unprotonated form from solution, then K_i should be corrected to reflect its changing state of covalent hydration as a proton is lost from the amino group. The present results suggest that the equilibrium constant for hydration of the neutral species is in the neighborhood of 17. If the aldehyde is bound unhydrated, then the true K_i of the free carbonyl compound is correspondingly less than the apparent lower limiting value of approximately 1.3×10^{-7} M at high pH, indicated by extrapolation of the data in Figure 1.

One possible explanation for the tight binding of inhibitory aldehydes by LAP would be that their space-filling requirements are less demanding than those of substrates; in addition, aldehydes are much more easily extracted from water than are the reactants or products of peptide hydrolysis (Wolfenden, 1983). However, the near-unity effect of deuterium substitution on the affinity of LAP for leucinal seems to exclude the possibility that this inhibitor is bound intact [see Lewis & Wolfenden (1977b), Figure 2].

Instead, it seems probable that aldehydes are bound as complexes formed by covalent addition of sulfur or oxygen. Information about the active site of LAP is limited, but there appears to be no compelling evidence of an essential sulfhydryl group (DeLange & Smith, 1971). Instead, the results appear to be most readily explained by supposing that leucinal is bound as an oxygen adduct: either the *gem*-diol that represents its most abundant form in free solution or an adduct formed by reaction with an alcoholic amino acid side chain at the active site. If the inhibitor is bound as a *gem*-diol, this could be considered to resemble an intermediate in direct attack by water on the scissile peptide bond of a bound substrate. A hemiacetal, on the other hand, might resemble intermediates in the formation and breakdown of an acyl-enzyme, if the normal reaction proceeds by double displacement. A similar ambiguity exists in the inhibition of carboxypeptidase A by substrate-related aldehydes (Galardy & Kortylewicz, 1984). An experimental distinction between these modes of binding would be helpful in deciding between these mechanisms of catalysis.

Registry No. LAP, 9001-61-0; L-leucinal, 82473-52-7; L-leucine *p*-nitroanilide, 4178-93-2, aminoacetaldehyde dimethyl acetal hydrochloride, 56766-81-5; aminoacetone hydrochloride, 7737-17-9; deuterium, 7782-39-0.

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