Noncompetitive, Reversible Inhibition of Aminoacylase-1 by a Series of L-α-Hydroxyl and L-α-Fluoro Fatty Acids: Ligand Specificity of Aspergillus oryzae and Porcine Kidney Enzymes

Takashi Tamura, Yoshiko Oki, Atsuhito Yoshida, Takatoshi Kuriyama, Hiroshi Kawakami, Hiroyuki Inoue, Kenji Inagaki, and Hidehiko Tanaka

Department of Bioresources Chemistry, Faculty of Agriculture, Okayama University, 1-1-1, Tsushima-naka, Okayama 700-8530, Japan

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L-Lactate and L-β-phenyllactate have been identified in the culture broth of Streptomyces sp. KY-11 as reversible noncompetitive inhibitors of Aspergillus oryzae aminoacylase-1 and porcine kidney aminoacylase I. A series of α-hydroxyl acids (DL-R-CH(OH)-COOH, R = Et, n-pro, n-butyl, n-pentyl, n-hexyl) also inhibited the two enzymes in reversible noncompetitive kinetics, and the inhibition potency (−log Ki) increased with the increased hydrophobicity of the R group. The two eukaryotic enzymes showed distinct preferences to the ligand α-alkyl group, and the fungus enzyme was inhibited by L-β-phenyllactate (R = benzyl) 103-fold more potently than the mammalian enzyme. L-α-Fluoro-β-phenyl-propionate and its D-isomer were used to show that the L-configuration of the α-substituent was important for potent inhibition of both the enzymes. The fungus aminoacylase-1 steeply decreased the affinity to α-fluoro- and α-hydroxy-n-caproate as pH was raised from 7 to 11, whereas the mammalian enzyme retained the affinity to these ligands under alkaline conditions. These results suggest that A. oryzae aminoacylase-1 has an acidic residue that interacts with -OH or -F, while the mammalian enzyme would have a basic residue that recognizes the α-substituents.

Key Words: aminoacylase-1; L-α-hydroxyl fatty acids; L-α-fluoro fatty acids; noncompetitive inhibitor.

Antibiotics can exert their antibacterial specificity by inhibiting bacteria-specific cellular devices such as the peptidoglycan biosynthesis and 70S ribosome-dependent protein synthesis. In contrast, specific antifungal agents must seek out subtle structural differences in closely related eukaryotic enzymes, and thereby exhibit the selective toxicity. Fundamental studies on protein structure and the ligand specificity thereof may provide lucid insight for designing specific enzyme inhibitors for the control of fungi and yeast. Aminoacylase-1 (EC 3.5.1.14) is a homodimeric zinc-binding metalloenzyme that catalyzes the decylation of various N-acylated L-amino acids, and the enzyme also serves as a potent peptidase with low substrate specificity (1, 2). Aminoacylase-1 has been found in high activity in a wide variety of organisms including microorganisms (3, 4), plants (5), and mammals (6, 7). Aspergillus oryzae produces the enzyme in large amounts, and the enzyme appears to play an integral role in nitrogen assimilation. There has been an increasing number of cases of fungal infection in compromised hosts with decreased immunity. A specific inhibitor of aminoacylase-1 may have potential pharmacologic roles. The present study was undertaken to discover specific inhibitors of A. oryzae aminoacylase-1 with low affinity to the mammalian counterpart enzyme. We have identified L-lactate and L-β-phenyllactate as inhibitors in the culture broth of Streptomyces sp. KY-11, which has been isolated from a soil sample of our university campus. The ligand specificity of A. oryzae aminoacylase-1 and the mammalian counterpart, aminoacylase-1, has been examined using a series of short-chain fatty acids bearing an α-hydroxyl or...
α-fluoro substituent. Inhibition by these ligands were fully reversed when enzyme-ligand complex was dialyzed against metal-free buffer, suggesting that inhibition was not caused by removal of the zinc ion of the enzyme.

MATERIALS AND METHODS

Materials. A. oryzae aminoacylase-1 was from Amano Pharmaceutical Co., Ltd. Porcine kidney aminoacylase I was purchased from Bioenzyme. Sodium salts of glycolate, DL-α-hydroxy-n-butyrate, DL-β-hydroxy-n-butyrate, DL-α-hydroxy-n-caproate, and DL-α-hydroxyisobutyrate were purchased from Nacalai Tesque. Sodium DL-α-hydroxy-n-valerate, sodium L-β-phenyllactate, and N-acetyl-L-α-aspartate were from Sigma. L-α-Fluoro-β-phenylpropionate and the β-isomer were synthesized from L- and α-phenylalanine, respectively, by the method of Olah et al. (8). DL-α-Fluoro-n-caproate and L-α-fluoroscaproate were also synthesized from DL-norleucine and L-leucine, respectively. Porcine kidney aminoacylase II was from ICN Biochemicals.

Aminoacylase-1 inhibition assay. A solution (800 μL) containing 94 mM Britton–Robinson buffer (pH 8.0), 0.25 mM CoCl₂, and 20 mM 2 aminoacylase-1 was mixed with 100 μL of sample solution, and the resulting mixture was incubated at 30°C for 5 min. The enzyme reaction was started by adding 100 μL of 500 mM N-acetyl-DL-methionine. After incubation at 30°C for 30 min, the solution was mixed with 200 μL of 25% (w/v) trichloroacetic acid, and precipitated protein was removed by centrifugation at 8000 rpm for 10 min.

RESULTS AND DISCUSSION

Aminoacylase-1 inhibitors from Streptomyces sp. KY-11. The yields of inhibitors I and II were 30 and 1 mg from 10 L of the culture broth, respectively. They were identified as lactate and β-phenyllactate on the basis of the following physicochemical properties. Inhibitor I, [1H NMR (D₂O), δ 1.2 (3H, d, J α = 6.95 Hz), 4.0 (1H, q, 6.95 Hz); 13C NMR (D₂O), δ 20.3, 67.6, 180.4 ppm; IR spectrum (KBr, cm⁻¹), 3300, 1180, 1720; EI-mass, m/z = 91 (M + 1). Inhibitor II, [1H NMR (D₂O), δ 3.0 (2H, m), 7.5 (5H, m); IR spectrum (KBr, cm⁻¹), 3300, 1700, 800, 700]. Inhibitor II was treated with diazomethane for mass spectrometry, producing the mass peaks (m/z) at 91 (benzyl), 163 (M – OH), and 181 (M + 1). Lactate isolated from the culture broth showed little optical rotation, and it served as a substrate for L- and D-lactate dehydrogenases. β-Phenyllactate from the culture broth showed little optical rotation, too. Since β-phenyllactate was not a substrate for the lactate dehydrogenases, commercially available L- and DL-β-phenyllactates were assayed for aminoacylase-1 inhibition. The L-isomer (IC₅₀ = 17.5 μM) was twice as inhibitory as the racemic form (IC₅₀ = 35.0 μM), suggesting that only the L-isomer can inhibit the catalysis. Double-reciprocal plots of 1/v against 1/[substrate] at different inhibitor concentrations gave straight lines with the same Kᵣ value, suggesting that the inhibition of A. oryzae aminoacylase-1 was irreversible noncompetitive with the substrate (Fig. 1). Apparent Kᵣ values of L-lactate and L-β-phenyllactate were estimated to be 0.9 mM and 17 μM, respectively, from Dixon plot of [inhibitor] versus 1/v. L-β-Phenyllactate and L-lactate also showed noncompetitive inhibition kinetics with porcine kidney aminoacylase I, with apparent Kᵣ values of 14.9 and 45.0 mM, respectively. Porcine kidney aminoacylase II, assayed with
N-acetyl-L-aspartate as the substrate, was not inhibited by L-lactate or L-β-phenyllactate.

Effects of α-alkyl group on inhibition potency. Because L-lactate and L-β-phenyllactate were α-hydroxyl acids, a series of α-hydroxyl fatty acids (DL-R-CH(OH)-COOH, R = H, Et, n-pro, ..., n-hexyl) was assayed for the inhibition potency on the fungus and mammalian aminoaoylases. Good correlations were observed between the inhibitory activity (−log $K_i$) and the hydrophobicity parameter (II) of the α-alkyl groups (11) (Fig. 2). Good correlations were also observed when parameters of molar volume were employed for the alkyl substituents (data not shown). The inhibition activity of a branched-chain α-hydroxyl acid was weak on both the enzymes; DL-α-hydroxyisocaproate showed 50-fold less inhibition than DL-α-hydroxy-n-caproate (Table I). DL-β-Hydroxy-n-butyrate and α-hydroxyisobutyrate were not inhibitory to the two enzymes while their constitutive isomer, DL-α-hydroxy-n-butyrate, was a moderate inhibitor of A. oryzae enzyme ($K_i = 1.2$ mM) and of the porcine enzyme ($K_i = 15$ mM). Besides the similar ligand specificity, the two enzymes also demonstrated distinctive preferences with respect to the α-alkyl group. (i) The mammalian enzyme showed lower affinity to all the inhibitors than did the fungus enzyme, and glycolate ($K_i = 5.0$ mM for A. oryzae aminoacylase-1) did not inhibit the porcine kidney en-

TABLE I

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Apparent $K_i$ (mM) A. oryzae enzyme</th>
<th>Porcine enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-α-Hydroxy-n-caproate</td>
<td>0.024 ± 0.009</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>DL-α-Fluoro-n-caproate</td>
<td>0.32 ± 0.04</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td>DL-α-Hydroxyisocaproate</td>
<td>1.50 ± 0.40</td>
<td>12.7 ± 4.2</td>
</tr>
<tr>
<td>L-α-Hydroxyisocaproate</td>
<td>0.65 ± 0.16</td>
<td>7.7 ± 1.8</td>
</tr>
<tr>
<td>L-α-Fluoroisocaproate</td>
<td>27.7 ± 1.3</td>
<td>28.8 ± 2.0</td>
</tr>
<tr>
<td>Isocaproate</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>L-β-Phenyllactate</td>
<td>0.017 ± 0.003</td>
<td>14.9 ± 1.4</td>
</tr>
<tr>
<td>DL-β-Phenylactate</td>
<td>0.025 ± 0.003</td>
<td>26.2 ± 6.1</td>
</tr>
<tr>
<td>L-α-Fluoro-β-phenylpropionate</td>
<td>0.77 ± 0.06</td>
<td>6.5 ± 0.9</td>
</tr>
<tr>
<td>DL-α-Fluoro-β-phenylpropionate</td>
<td>3.4 ± 0.6</td>
<td>34.6 ± 2.6</td>
</tr>
<tr>
<td>β-Phenylpropionate</td>
<td>5.3 ± 0.7</td>
<td>n.i.</td>
</tr>
</tbody>
</table>

$^a$ Errors are the standard error of three measurements.

$^b$ n.i., not inhibitory.
zyme. (ii) The fungus enzyme was most strongly inhibited by DL-α-hydroxy-n-octanoate (R = n-C6H13; $K_i = 11 \ \mu M$) among the compounds tested, while the mammalian enzyme was most potently inhibited by DL-α-hydroxy-n-caproate (R = n-C4H9; $K_i = 0.26 \ \mu M$). (iii) The porcine kidney enzyme showed an exclusive preference to straight alkyl chains while the fungus enzyme was capable of binding a benzyl group as well. This was illustrated by the results that A. oryzae aminoacylase-1 showed similar affinity to DL-β-phenyllactate ($K_i = 25 \ \mu M$) and DL-α-hydroxy-n-caproate ($K_i = 24 \ \mu M$), while porcine kidney aminoacylase I showed 100-fold higher affinity to DL-α-hydroxy-n-caproate ($K_i = 0.26 \ \mu M$) over DL-β-phenyllactate ($K_i = 26.2 \ \mu M$). Thus, the two eukaryotic enzymes, showing similar inhibitor specificity to α-hydroxyl fatty acid, probably differ in the size of the hydrophobic pocket, and this difference allowed L-β-phenyllactate to inhibit the fungus enzyme 10-fold more potently than the mammalian enzyme.

Effects of α-substituents on inhibition potency. α-Hydroxyl group appeared to be another structural key element for a ligand to bind potently to these enzymes since various straight-chain fatty acids (C3 to C10) bearing no α-hydroxyl group showed little inhibitory activity to both the enzymes. The α-hydroxyl group would contribute to lower the carboxyl $pK_i$, as well as introducing hydrogen-bonding interactions in the ligand-binding site. The latter effect of hydrogen bonding may be relevant to the observation that the stereochemistry of α-hydroxyl group was important for the inhibition. L-Lactate inhibited the A. oryzae enzyme ($K_i = 0.9 \ \mu M$) and the porcine kidney enzyme ($K_i = 45.0 \ \mu M$), but α-lactate was not inhibitory to either enzyme. L-α-Hydroxyisocaproate and l-β-phenyllactate had $K_i$ values that were almost half of those of their racemate, suggesting that only the l-isomers were inhibitory to both the enzymes (Table I). The conclusion that l-configuration is essential for the potent inhibition also indicates that the inhibitor-binding step involves orienting alkyl and hydroxyl groups properly in the ligand-binding site, and this functionality orientation would be assisted by ionic interactions to the inhibitor’s carboxyl group. To characterize the putative hydrogen bonding implementing this stereospecificity, L-α-fluoro-β-phenylpropionate and its d-isomer were stereospecifically synthesized, and their apparent $K_i$ values were assayed. L-α-Fluoro-β-phenylpropionate showed several-fold higher affinity to the fungus enzyme than the d-α-fluoro enantiomer and β-phenylpropionate (Table I). Presumably, L-α-fluorine was involved in the hydrogen-bonding interaction in a fashion analogous to that of the L-α-hydroxyl group of l-β-phenyllactate. The d-α-fluoro enantiomer may not conform to the hydrogen bonding in the ligand-binding site, and it may merely contribute to lower the carboxyl $pK_i$; thus the d-α-fluoro analogue showed a slightly smaller $K_i$ value than the corresponding bald acid, β-phenylpropionate. It should be pointed out, however, that a hydroxyl group is capable of serving as a hydrogen donor and a hydrogen acceptor simultaneously in a hydrogen-bonding network, but fluorine can serve only as a hydrogen acceptor. It was therefore anticipated that a fluorine analog might show less potent inhibition than the original α-hydroxy acid. In fact, L-α-fluoro-β-phenylpropionate was bound to A. oryzae aminoacylase-1 with 43-fold lower affinity than L-β-phenyllactate. L-α-Fluoro-isocaproate showed 45-fold lower affinity to the fungus enzyme compared to the corresponding hydroxy acid (Table I). For porcine kidney aminoacylase I, however, the affinity decrease by fluorine substitution was only several to 10-fold. In the case of β-phenyllactate, the substitution by fluorine even decreased the apparent $K_i$ value from 14.9 to 6.5 mM. Accordingly, we have hypothesized that the distinct effects of OH to F replacement might result largely from differences in the basicity of hydrogen-bonding residues. We next examined the effect of pH on the inhibition potency to better define the putative functional group that recognizes the α-hydroxyl or α-fluoro moiety in the ligand-binding site. DL-α-Hydroxy-n-caproate, to which the mammalian enzyme showed the highest affinity, and its fluorine analog were used for this investigation. When the pH was increased from 7.0 to 11.0, A. oryzae aminoacylase-1 decreased the binding potency to the α-hydroxyl or α-fluoro inhibitors, whereas porcine kidney aminoacylase I exhibited little change in the affinity to either of the ligands3 (Fig. 3). From these results and the observation that α-fluoro-n-caproate decreased the affinity to the fungus enzyme more steeply than the α-hydroxyl ligand, we presumed that A. oryzae aminoacylase-1 has an acidic residue for the hydrogen bonding, while the corresponding residue might instead be a basic one in the mammalian enzyme.

Inhibition mechanism. It has been reported that a potent metal chelator, o-phenanthroline, inhibits A. oryzae aminoacylase-1 in a noncompetitive mode (12, 13). The metal chelator inactivates the enzyme in a time-dependent, multiphasic process involving a rapid, reversible o-phenanthroline-binding step followed by removal of the metal, thus leading to irreversible inactivation (14, 15). Zinc ion of aminoacylase-1 is considered to play primarily a structural role, stabilizing the active conformation of the enzyme but not participat-

3 The optimal pH for the reaction of N-acetyl-l-methionine was at 8.0 for both the enzymes. At pH higher than 9, inhibition assay was carried out under the same conditions as at pH 8 except for prolonged incubation time up to 40 min, where the enzyme reaction was linear with respect to the incubation time.
ing actively in catalysis (16). It is unlikely though that the noncompetitive inhibition by α-hydroxyl and α-fluoro fatty acids arises from removal of the zinc ion for several reasons. First, these inhibitors did not show time-dependent inactivation, which is characteristic of the inactivation by metal chelator. Second, our inhibition assay has been carried out in the presence of 0.20 mM CoCl₂. If the inhibitors had high affinity for metals, they would be sequestered by free cobalt ion present in the large excess over the enzyme concentrations. Last, the enzyme recovered the full activity when enzyme–ligand complexes were thoroughly dialyzed against metal-free 50 mM Tris–HCl buffer, pH 8 (Table II). As previously reported, inactivation by o-phenanthroline was not reversed with dialysis against metal-free buffer under the same conditions. Because L-α-hydroxy or L-α-fluoro fatty acids resemble some L-α-amino acids, such as L-methionine and L-phenylalanine, these ligands might inhibit the enzyme by fitting itself in substrate-binding site of free enzyme or of E–P complex. However, since aminoa-

FIG. 3. Effect of pH on the inhibition potency of DL-α-hydroxy- and DL-α-fluorocaproate. Kᵢ values were assayed in Britton-Robinson buffer at the designated pH. Kᵢ values of DL-α-hydroxy-n-caproate (circles) and DL-α-fluoro-n-caproate (triangles) were assayed for A. oryzae aminoacylase-1 (closed symbols) and porcine kidney aminoacylase I (open symbols).

ACKNOWLEDGMENTS
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TABLE II
Recovery of Aminoacylase-1 Activity by Dialysis of the Enzyme-Ligand Complex

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc (mM)</th>
<th>Inactivation* (mU)</th>
<th>Recovery* (mU)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>0.00</td>
<td>79.8 ± 3.8</td>
<td>86.3 ± 4.8</td>
<td>100</td>
</tr>
<tr>
<td>o-Pheanthroline</td>
<td>1.30</td>
<td>0.96 ± 1.4</td>
<td>1.2</td>
<td>11.4</td>
</tr>
<tr>
<td>L-β-Phenyllactate</td>
<td>0.87</td>
<td>8.8 ± 2.9</td>
<td>11.0</td>
<td>11.8</td>
</tr>
<tr>
<td>L-α-Fluo-β-phenylpropionate</td>
<td>7.80</td>
<td>7.3 ± 2.4</td>
<td>86.4 ± 1.2</td>
<td>100</td>
</tr>
</tbody>
</table>

* The activity of aminoacylase-1 in the presence of a noncompetitive inhibitor.

* The inhibitor was removed from enzyme by thorough dialysis against 50 mM Tris–HCl buffer (pH 8.0), and then the enzyme activity was assayed.

* The enzyme activity of the control experiments without inhibitor was considered to be 100%.
REFERENCES